Regulation and function of hemoglobin in barley aleurone tissue

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Xianzhou Nie

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REGULATION AND FUNCTION OF HEMOGLOBIN IN BARLEY ALEURONE TISSUE

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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List of Abbreviations

AA	Antimycin A
ABA	Abscisic acid
ADH	Alcohol dehydrogenase
Adh	Alcohol dehydrogenase gene
ADP	Adenosine 5'-diphosphate
ANP	Anaerobic polypeptide
ATP	Adenosine 5'-triphosphate
BHAM	Benzhydroxamic acid
CaM	Calmodulin
CHI	Cycloheximide
4,6-D	4,6-Dioxoheptanoic acid
dCTP	deoxycytidine 5'-triphosphate
DFOX	desferrioxamine
DNP	2,4-Dinitrophenol
EDTA	Ethylenediaminetetraacetoc acid
EGTA	Ethylene glycol-bis-N,N,N',N'-tetraacetic acid
EPO	Erythropoietin
Epo	Erythropoietin gene
ER	Endoplasmic reticulum
GA	Gibberellin

GTP	Guanosine 5'-triphosphate
h	hours
Hb	Hemoglobin
kD	kilodaltons
kb	kilobase pairs
Lb	Leghemoglobin
LDH	Lactate dehydrogenase
Ldh	Lactate dehydrogenase gene
Mb	Myoglobin
NADH	Nicotinamide adenine dinucleotide (reduced)
OA	Okadaic acid
PAGE	Polyacrylamide gel electrophoresis
PDC	Pyruvate dehydrogenase
Pdc	Pyruvate dehydrogenase gene
RR	Ruthenium red
SDS	Sodium dodecyl sulfate
VHb	*Vitreoscilla* hemoglobin
vgb	*Vitreoscilla* hemoglobin gene
$\Delta\mu H^+$	Proton gradient

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Abstract

Hemoglobin genes have been found in cereal plants including barley, rye, maize and wheat, and a Hb cDNA has been isolated from a barley cDNA library (Taylor et al., 1994). In this study, a barley Hb cDNA was demonstrated to also hybridize to genomic DNA sequences in rice, oat and bean, suggesting that barley Hb-like DNA sequences are widely distributed in the plant kingdom. The expression of Hb gene(s) in barley is tissue specific under normal growth conditions. Transcripts were found in roots, coleoptiles and aleurone layers but not in leaves. The gene, along with alcohol dehydrogenase and lactate dehydrogenase, was induced by oxygen deprivation in barley aleurone layers. Both Hb transcripts and protein were enhanced by anaerobiosis in barley aleurone layers and coleoptiles. However, the induction of protein was not as pronounced as that of mRNA. Supplying oxygen to oxygen deficient tissue decreased both Hb mRNA and protein, indicating that the gene expression is oxygen dependent.

The effect of a number of respiratory inhibitors on barley aleurone layers has been examined to determine how increased hemoglobin gene expression occurs. Carbon monoxide induced Hb expression. Cyanide and antimycin A, two mitochondrial respiratory inhibitors, reduced oxygen consumption and, at the same time, strongly enhanced Hb messenger RNA levels. Treatment with the oxidative phosphorylation uncoupler 2,4-dinitrophenol markedly increased oxygen consumption and had a similar positive effect on Hb gene expression, suggesting that the expression of hemoglobin is not directly related to oxygen usage in the tissue. Hb transcription was also stimulated by the oxidative phosphorylation inhibitor oligomycin. The results suggest that the level of ATP may be critical in the induction of Hb gene transcription.

The signal transduction pathway leading to Hb gene expression in barley aleurone layers has been investigated. Ruthenium red, an organelle calcium channel blocker, inhibited anoxia-induced Hb transcription in a dose-dependent manner. The divalent ionophore, A23187, combined with EGTA dramatically reduced anoxia-induced Hb transcripts. The normal response of Hb to anoxia was restored by adding exogenous Ca^{2+} but not Mg^{2+}. The results indicate that cytosolic calcium is involved in Hb gene regulation. W-7, a calmodulin antagonist, appeared not to affect anaerobically induced Hb mRNA even though it could induce Hb in normoxia, suggesting that calmodulin is not required in anaerobic induction of Hb in barley aleurone layers. Okadaic acid, a protein phosphatase inhibitor, was shown to abolish anaerobic-induced Hb transcripts dramatically, whereas A3, a protein kinase inhibitor, failed to do this. The results suggest that an okadaic acid-sensitive protein phosphatase is involved in the signal transduction pathway leading to Hb induction. Interestingly, both A3 and okadaic acid stimulated Hb expression under normoxia. The protein synthesis inhibitor, cycloheximide, blocked Hb gene expression, indicating that *de novo* synthesis of protein is required in Hb gene expression.

It is unclear yet whether this protein functions as an oxygen carrier or a redox protein. However, it is unlikely that the protein acts as an oxygen sensor since: (1) Hb expression occurred parallel to, rather than preceded, *Adh* and *Ldh* expression; (2) Blocking heme biosynthesis by incubating barley aleurone layers with heme biosynthesis inhibitors 4,6-dioxoheptanoic acid and desferrioxamine failed to affect *Adh* and *Ldh*; (3) Carbon monoxide (CO)-Hb shares the same conformation with oxy-Hb. If deoxy-Hb is the active form to trigger anaerobic responses (Goldberg et al., 1988), CO-Hb would thus lock Hb in its inactive form. However, CO treatment did not inhibit hypoxia induced *Adh* and *Ldh*, instead, it stimulated both genes under anaerobiosis.

1. Objectives of the Research

Non-symbiotic plant hemoglobins have been isolated and characterized in several plant species including both monocots and dicots. However, little is known about the physiological functions and regulation of these molecules in plants. The major objective of this research is thus to understand the physiological significance and also the regulation of hemoglobin in barley, particularly in barley aleurone tissue.

To gain the goal, it would be essential to investigate the general pattern of the gene expression, i.e., in what tissue(s) and under what condition(s) the gene is expressed.

Since a Hb cDNA was isolated from a barley aleurone cDNA library, barley aleurone tissue would thus be the first candidate to conduct the research. Unlike the adult barley plant, barley aleurone layers can bear prolonged anoxia with active ethanol and lactate fermentation. Does Hb respond to anaerobiosis similarly as alcohol dehydrogenase and lactate dehydrogenase? To answer this question, it would be beneficial to analyse the expression of the Hb gene and gene product in response to the stress.

Although it is well known that Hb is induced by O_2 deficit in both prokaryotes and eukaryotes including *E.coli*, *Vitreoscilla*, yeast, barley and maize, the mechanism of the induction has yet to be characterized. Since Hb shows reversible O_2 binding, and O_2 availability directly affects respiration of organisms, it would be interesting to investigate the relationship between Hb gene expression and respiration in barley aleurone layers.

Signal transduction has attracted a lot of attention in various organisms. It appears that the

cytosol calcium, calmodulin and reversible protein phosphorylation catalysed by protein kinases and phosphatases are involved in many signal transduction pathways. To understand the regulation of Hb gene expression in barley aleurone layers, it would be useful to study the possible signal transduction components involved in the gene regulation.

The non-symbiotic plant hemoglobin has been proposed to function as an oxygen carrier, an oxygen sensor, or a terminal oxidase. However, the major role of Hb in plants remains unclear. Thus, to understand the physiological function(s) of Hb would be one of the most important aspects of the research, which may have significant impact on our understanding about the survival of plants under anaerobiosis.

2. Literature Review

2.1. Hemoglobin

2.1.1. Introduction

The common preconception about hemoglobin (Hb) is that the protein occurs exclusively in vertebrates transporting oxygen to tissues for respiration. The existence of the protein in plant sources was not recognized until 1939 when Kubo noticed a red pigment, which was later identified as an animal-hemoglobin like protein, leghemoglobin, in N_2-fixing nodules in legume plants (Kubo, 1939). This protein had been thought to be accidentally transferred from an animal source during the evolution process (Appleby, 1984;1985; 1992; Hardison, 1996). However, such an assumption was challenged by the discovery of non-legume source hemoglobins in dicotyledonous plants even though some of these plants are able to nodulate with rhizobium (Landsman et al., 1986; Fleming et al., 1987). The recent isolation and identification of Hb in barley and other cereal plants (Taylor et al., 1994) strongly support the suggestion that Hb occurs widely in the plant kingdom (Appleby et al., 1983). Moreover, a non-symbiotic hemoglobin which is expressed widely in different tissues, was recently found in soybean (Andersson et al., 1996) as predicted previously (Appleby, 1992; Taylor et al., 1994). Thus, it is now generally accepted that hemoglobin is widely distributed in plants, maybe the whole plant kingdom. However, little is known about the functions of non-symbiotic Hb in plants. In this review, I will discuss various aspects of hemoglobin with the emphasis on plant hemoglobins.

2.1.2. General aspects of hemoglobin

Hemoglobins are proteins in which a heme group is non-covalently attached to a globin

(polypeptide) molecule. They are distinguished from non-hemeproteins by their red colour. The red blood cell hemoglobins of vertebrates are tetramers containing two α and two β subunits, each is comprised of a heme and a globin chain. Vertebrate muscle hemoglobin (myoglobin) is monomeric protein which contains only a heme group and a globin chain (Voet & Voet, 1990). Plant hemoglobins described so far are either monomers or homodimers without heme-heme interaction (Wittenberg & Wittenberg, 1990; Duff et al., 1997). Like some hemeproteins, hemoglobins, including myoglobin, possess two distinct conformations, an oxy-form and an deoxy-form, which show distinct absorbance spectra. The oxy-Hb has two absorbance peaks at 540 and 574 nm, whereas the deoxy-Hb shows one absorption peak located at 557 nm (Appleby, 1992).

It is well known that hemoglobins reversibly associate with oxygen regardless of their origin and subunit structure. However, different hemoglobins may differ in their oxygen affinity as shown in the Table I.

Table I. ***Oxygen affinity of various hemoglobins***

Hb	O_2 affinity (nM)	References
Soybean (Lb)	48	Gibson et al., 1989
Parasponia	90	Wittenberg et al., 1986
Casuarina	135	Fleming et al., 1987
Barley	2.86	Duff et al., 1997
Vitreoscilla	6000	Dikshit & Webster, 1988
Yeast	20	Oshino et al., 1973

Environmental factors, such as pH, organic phosphate, CO_2 can affect their oxygen affinity (Komiyama et al., 1995; Giardina et al., 1995). Hemoglobin concentration can also affect the oxygen affinity in intact cells (Airaksinen & Nikinmaa, 1995).

Certain small molecules, such as CO, NO and H_2S, bind to the sixth liganding position of the Fe(II) in hemoglobin with much higher affinity than does O_2. This as well as their similar binding to the hemes of cytochromes accounts for their highly toxic properties (for reference, see Voet & Voet, 1990).

2.1.3. Distribution of Hb in nature

Hemoglobins are now believed to be widely distributed in nature ranging from bacteria, yeast, molds, most phyla of higher invertebrates, protochordates to vertebrates and higher plants. The best known hemoglobins are vertebrate Hbs and myoglobins. Vertebrate Hbs are encoded by α-globin and β-globin genes, which show high similarities (for reference, see Hardison, 1996). The amino acid sequences of the α-globins and β-globins are about 50% identical (Hardison, 1996), no matter what vertebrate species is the source, indicating that these Hbs have a common ancestor. Myoglobin differs from both α- and β-globins by existing as a monomeric form (Wittenberg & Wittenberg, 1990). However, myoglobin shows high similarities with both α- and β-globins in its three-dimensional structure, its primary amino acid and nucleotide sequences (for reference, see Voet & Voet, 1990). Moreover, hemoglobins found in invertebrates are clearly related to those of the vertebrates (Sherman et al., 1992).

Since the first discovery of Hb in plants, this molecule has been found in various plant species ranging from leguminous to non-leguminous dicots to monocots. Nodule specific

hemoglobins (leghemoglobins, Lbs) are the best-known plant Hbs. Although Lbs are distinct from vertebrate Hbs and myoglobins by their primary sequences, *e.g.*, 80% of the amino acid residues are different to those of vertebrates (for reference, see Hardison, 1996), the three-dimensional structures, ligand-binding properties and absorption spectrum of Lbs show extraordinary high similarities to those of vertebrates, indicating that they belong to the same family (Appleby, 1992). Besides leghemoglobins, hemoglobins which are distinct from Lbs were found in root nodules of the non-leguminous plant *Parasponia andersonii* (Appleby et al., 1983). These proteins may play a role in nitrogen fixation very similar to their counterparts in legumes. However, *Parasponia* Hb is also observed to have a nonsymbiotic role in *Parasponia* roots (Appleby et al., 1988). The discovery of barley hemoglobin and its presence in cereal plants expands our understanding about hemoglobin in plants (Taylor et al., 1994), supporting the assumption that Hb exists widely in the plant kingdom.

Hemoglobins have also been observed in yeast (Oshino et al., 1973) and bacteria (Dikshit et al., 1989). A flavohemoglobin from the yeast is a fusion of a heme-binding domain and FAD-binding domain (Zhu & Riggs, 1992). Very similar structure is found in bacteria Hbs (LaCelle et al., 1996; Poole et al., 1996). However, the primary sequences of yeast and bacteria hemoglobins are different from those of plant and animal hemoglobins (Zhu & Riggs, 1992).

2.1.4. Plant hemoglobin

2.1.4.1. Legume hemoglobins

2.1.4.1.1. Symbiotic hemoglobins

Leguminous plants have the capacity to form a symbiosis with *Rhizobium*, producing

nodules which could fix atmospheric nitrogen to supply its host plant. The key enzyme in the N_2 fixation process is nitrogenase, an enzyme which is very sensitive to free oxygen. Hemoglobins play a crucial role to allow the system to meet the oxygen requirement for bacteroid respiration while keeping oxygen tension low enough to allow nitrogenase to function (Appleby, 1984). It was proposed that the hemoglobin in the nodules was from the bacteria rather than the host plants due to its apparent absence in other parts of the plants (Appleby, 1984). Further research strongly indicated that the legume-hemoglobin is indeed from the host plants since Lb apoproteins were synthesized in a wheat germ system by using poly(A)-containing mRNA isolated from soybean nodules as templates (Verma et al., 1974). Moreover, purified Lb-cDNA prepared from such mRNA would hybridize with soybean DNA but not with *Rhizobium* DNA (Sidloi-Lumbroso et al., 1978).

There are several isoforms of leghemoglobin in legume plants. In soybean, two major forms which are named Lb*a* and Lb*c* and two minor ones which are named Lb*b* and Lb*d* have been isolated. Further studies have revealed the presence of four major forms, Lb*a*, Lb*c*1, Lb*c*2 and Lb*c*3, which represent separate gene products (Fuchsman & Appleby, 1979), and four minor forms, Lb*b*, Lb*d1*, Lb*d2* and Lb*d3* arising from post-translational acetylation of the four major forms (Appleby, 1992).

Lbs are highly nodule-specific. Uninfected plant cells do not contain any trace of Lb (Bisseling et al., 1983), thus signals for the Lb gene expression appear to be under control of *Rhizobium* infection. Studies on the promoter regions indicate that two motifs are critical for nodule specific expression, *i.e.*, the nodulin box 5'-AAAGAT-3' and 5'-CTCTT-3', separated by six to seven nucleotides (Ramlov et al., 1993).

Soybean Lb genes have three introns (intervening sequences), of which two are at exactly the same positions found for animal globin genes. The third, the central intron, is missing from modern animal globins but is present in the soybean Lb genes in the position which had been predicted by computational analysis of animal globin gene structures before the intron was identified in Lb genes (Itylding-Nielsen et al., 1982).

2.1.4.1.2. Non-symbiotic hemoglobin

A non-symbiotic hemoglobin gene was identified in soybean recently (Andersson et al., 1996). The existence of non-symbiotic hemoglobin in legumes had been predicted for several years (Appleby, 1988; Taylor et al., 1994). The impetus for pursuing the existence of a non-symbiotic legume Hb came from the discoveries of Hbs in non-symbiotic plants such as *Trema* (Bogusz et al., 1988) and monocot plants (Taylor et al., 1994). Like Lbs, this non-symbiotic Hb gene contains three introns, but in the promoter region, the critical nodulin motif 5'-CTCTT-3' is absent. Northern analysis shows that the gene is expressed in cotyledons, stems of seedlings, roots, young leaves and in some cells in the nodules that are associated with the N_2-fixing *Bradyrhizobium* symbiont (Andersson et al., 1996).

2.1.4.2. Hemoglobin in non-legume dicotyledonous plants

2.1.4.2.1. Symbiotic hemoglobin in non-leguminous plants

Parasponia hemoglobin was the first hemoglobin isolated from a non-legume source (Appleby et al., 1983). It was isolated from N_2-fixing nodules formed by association of *Rhizobium* with an nonleguminous plant, *Parasponia*. The protein consists of two identical polypeptide

chains of 155 amino acids and shows extensive sequence homology with Lbs (approximately 40 %) (Kortt et al., 1985). The genomic DNA sequence of *Parasponia* Hb contains three introns which are at the same positions as those in the leghemoglobin gene (Landsmann et al., 1986).

Actinorhizal plants, the species that are capable of participating in a symbiotic N_2-fixing relationship with actinomycetes of the genus *Frankia*, have been found to possess hemoglobin (Fleming et al., 1987) or Lb-like sequences (Roberts et al., 1985). These proteins are largely located in the nodules of the host (*Casuarina glauca)-Frankia*, remaining firmly bound to cell membranes. The oxygen affinity of *Casuarina* hemoglobin is lower than that found for any of the legume nodule hemoglobins, suggesting that the *Frankia* endophyte of *Casuarina* might be more oxygen-tolerant than are the *Rhizobium* endophytes of legume nodules (Fleming et al., 1987). High concentrations of a hemoglobin-like protein, later confirmed as a true hemoglobin (Pathirana & Tjepkema, 1995), have also been found in nodules of *Casuarina cunninghamiana* and *Myria gale* by using the 416 to 420 nm absorption band of carboxyhemoglobin to estimate the hemoglobin concentration (Tjepkema & Asa, 1987).

Unlike *Parasponia* in which the same Hb is expressed in both nodules and normal root tissues (both symbiotic and nonsymbiotic roles), *Casuarina* possesses two hemoglobin genes, one (symbiotic) is exclusively expressed in nodules, and the other (nonsymbiotic) is expressed in non-symbiotic root tissues (Jacobsen-Lyon et al., 1995; Christensen et al., 1991). Promoter analysis indicates that *Casuarina* symbiotic Hb contains nodulin motifs, *i.e.*, 5'-AAAGAT-3' and 5'-CTCTT-3' (Christensen et al., 1991), suggesting that these motifs have the same function in the hemoglobin genes of the *Casuarina-Frankia* symbiosis as they do in that of the legume-*Rhizobium* symbiosis. The *Casuarina* non-symbiosis Hb promoter contains sequences related to

but not identical to the nodulin motifs, and the spacing (4 bp) between the motif sequences differs from that in the symbiotic genes (Jacobsen-Lyon et al., 1995).

2.1.4.2.2. Non-symbiotic hemoglobin in non-legume dicots

Structural similarities amongst symbiotic hemoglobins and animal hemoglobins at the protein and gene levels indicated a possible common evolutionary origin (Hardison, 1996). This suggests that hemoglobins may be present in all plants. The finding of hemoglobin in *Trema tomentosa*, a non-nodulating relative of *Parasponia* (*Ulmaceae*), strongly supported the proposal. Like all the plant hemoglobin genes discussed above, the *Trema* Hb gene contains three introns located at positions identical to those of leguminous plant species (Bogusz et al., 1988). The expression of *Trema* Hb is tissue specific, mainly in the root tissue, which is similar to *Parasponia* Hb in non-nodulated plants. Promoter studies indicate that the promoters from *Trema* Hb and *Parasponia* Hb direct root-specific expression in transgenic tobacco (Bogusz et al., 1990).

2.1.4.3. Hemoglobins in monocotyledonous plants

The significant discovery of hemoglobin in monocotyledonous plants was achieved by an immunologically screen of an aleurone cDNA library with antiserum for limit dextrinase (Taylor *et al.*, 1994). The isolated cDNA has a strong sequence homology to *Parasponia* and *Trema* hemoglobins, encoding a predicted 162 amino acid polypeptide with a molecular weight around 18 kD. The predicted amino acid sequence shows 71% identity with *Parasponia* Hb, with a further 16% of residues being conservative replacements. However, the sequence is distinct from, but still shows extensive sequence homology (approx. 40% are identical, another 40% are

conservative) to leghemoglobins. More important, residues conserved between known dicots and animal Hbs are also conserved in barley Hb.

Southern blot analysis has revealed that the hemoglobin gene is present in all tested monocotyledonous plants, including barley, maize, rye and wheat (Taylor et al., 1994). RNA hybridization experiments show that the expression of Hb is tissue specific, mainly in roots, coleoptiles and aleurone layers. Hb messenger RNA in leaves and stems was not yet detected. Further study indicates that gene expression is associated with oxygen tension: when oxygen concentration decreases to a certain level, the gene is significantly induced (Taylor et al., 1994).

2.1.5. Functions of hemoglobin

The tetrameric vertebrate hemoglobins from erythrocytes have been known for centuries to transport oxygen from lungs, gills, or skin of some animals to the tissue where it is utilized as the terminal electron acceptor in catabolism (Giardina et al., 1995). Besides this basic function of oxygen transport, these tetrameric hemoglobins have other functions as well. For example, it has been suggested that human hemoglobin functions as a molecular heat transducer through its oxygenation-deoxygenation cycle, and as a modulator of erythrocyte metabolism (Giardina et al., 1995). It has also been proposed that the hemoglobin plays a role in erythrocyte senescence and malaria resistance (for references, see Giardina et al., 1995).

Unlike vertebrate erythrocyte hemoglobin, cytoplasmic hemoglobins, including myoglobin and plant hemoglobins, lack the sigmoid shape of oxygenation curve due to the absence of heme-heme interaction. The functions of myoglobin and plant hemoglobins, thus, differ from that of vertebrate erythrocyte hemoglobin. It has been proposed that the cytoplasmic hemoglobin may

facilitate oxygen diffusion (For reference, see Wittenberg & Wittenberg, 1990), or function as a mediator of oxidative phosphorylation (Wittenberg & Wittenberg, 1990), or even as a terminal oxidase or an oxygen sensor (Appleby et al., 1988). In this part of review, we will focus on the functions of cytoplasmic hemoglobin.

2.1.5.1 Hemoglobin mediated oxygen diffusion and oxygen storage

Hemoglobins have been found to function in states of partial oxygenation, *i.e.*, a certain percentage of hemoglobin including myoglobin is oxygenated in the tissue (Wittenberg & Wittenberg, 1990). In myoglobin-dependent systems, such as mammalian muscle and heart, free diffusion of O_2 inside the cell is extremely slow due to the thick cytoplasm and low solubility of oxygen in aqueous solution. Free oxygen pressure in mitochondria is at very low level in the range of 1 torr, whereas in erythrocyte it is around 20-25 torr (for reference, see Wittenberg & Wittenberg, 1990). The diffusion of O_2-bound myoglobin molecules generates a flux of oxygen in a gradient of oxygen pressure. It is estimated that myoglobin concentration in the heart muscle exceeds free O_2 concentration more than 30-fold (Wittenberg & Wittenberg, 1990), thus the diffusion of O_2-bound myoglobin is much more important than that of free O_2.

Leghemoglobin plays an important role in the N_2-fixation process. Atmospheric N_2 is fixed (reduced) by nitrogenase, an enzyme that is extremely sensitive to free O_2, in symbiotic bacteroids. Paradoxically, reduction of nitrogen to ammonia depends on a continuous supply of oxygen to support bacterial oxidative phosphorylation to meet the demand for ATP. To solve this problem, the bacteroids are separated from the cytoplasm of the host plant cell by a membrane where Lb may reach a concentration of 3.76 mM (Appleby, 1984). It has been estimated that with

20% oxygenation of Lb, the steady state concentration of free, dissolved O_2 at the bacteroid surface would be around 7-10 nM (Appleby, 1984). Thus, O_2-bound Lb is around 0.75 mM, which is about 75,000 times greater than that of free dissolved O_2, indicating the entire flux of oxygen to the bacteroids should be Lb facilitated (Appleby, 1992).

O_2 storage by myoglobin in aquatic mammals such as whales and seals is significant, leading to the ability of these animals to submerge for extended time. In these animals, myoglobin concentration is about ten times greater than that in terrestrial mammals (see Voet & Voet, 1990). Large amounts of oxygenated myoglobin formed during the merging period could be used later when O_2 levels in the erythrocyte decrease.

2.1.5.2. Oxygen sensing

In natural conditions, all organisms have to face unfavourable conditions such as drought, low temperature, flooding and so on. Mechanisms that favour an organism's survival have evolved. Anaerobic metabolism, which is discussed in a separate part in this review, is an adaptive mechanism applied in many organisms facing oxygen shortage. A molecule that senses such an oxygen deprivation has been proposed in different species from mammalian to bacterial systems (Acker, 1994). Some evidence suggests that the O_2 sensor is a hemeprotein (Gilles-Gonzalez et al., 1994; Goldberg et al., 1988; Acker, 1994; Poole et al., 1994).

Hemoglobin level in mammals is regulated by the hormone erythropoietin (Minegishi et al., 1994), which is significantly stimulated by hypoxia (Goldberg et al., 1987; 1988). In human hepatoma cells, erythropoietin gene expression is regulated by a heme protein since the gene expression is blocked by heme biosynthesis inhibitors. Moreover, CO, which maintains the

hemeproteins in their oxy-conformation, blocks the hypoxia-induced erythropoietin gene expression (Goldberg et al., 1988).

In bacterial systems, hemoglobin-related proteins are reported to act as oxygen sensors. *E. coli* hemoglobin contains a flavin-binding domain fused to the heme-binding domain (Andrews et al., 1992; Copper et al., 1994). The binding of oxygen by the heme domain may be accompanied by conformational change which acts as a switching mechanism to control activity of the second domain. Such a mechanism is also found in *Rhizobium meliloti* membrane-bound FixL, a hemeprotein. The FixL protein possesses a heme-binding oxygen-sensing domain (hemoglobin) and a functional C-terminal kinase domain (Monson et al., 1992). This protein senses oxygen through its heme moiety and transduces this signal by controlling the phosphorylation of Fix J, which, through a cascade mechanism, results in transmission of the "low oxygen" signal to genes responsible for the synthesis of proteins essential for nitrogen fixation (Gilles-Gonzalez et al., 1991).

Appleby et al. (1988) proposed that non-symbiotic hemoglobin of plants may serve as an oxygen sensor to monitor oxygen availability and thus to shift aerobic metabolism to anaerobic pathways in plant roots. Unlike that of leghemoglobin, the concentration of non-symbiotic hemoglobin in the cytoplasm may be too low to facilitate oxygen diffusion. However, this concentration should be sufficiently high to serve as an oxygen sensor .

2.1.5.3. Other functions

It has been proposed that cytoplasmic hemoglobin of yeast and bacteria function as terminal oxidases (Wittenberg & Wittenberg, 1990). The yeast and *E. coli* hemoglobin is a two-

domain protein with both heme and flavin prosthetic groups (Zhu & Riggs, 1992). The flavin domain of yeast hemoglobin is homologous with members of a flavoprotein family that includes ferredoxin reductase, nitric oxide synthase, and cytochrome P-450 reductase. Binding of oxygen by the heme domain may be accompanied by conformational change which acts as a switching mechanism to control the activity of the second domain (Zhu & Riggs, 1992).

Vitreoscilla Hb (VHb) is a dimer of identical subunits found in the obligate aerobic bacterium *Vitreoscilla*. VHb demonstrates a significant amino acid sequence similarity with eukaryotic globins (Wakabayashi et al., 1986). Intracellular expression of VHb in *E. coli* significantly improves *E. coli* ATP production and its growth under oxygen limited conditions (Khosla & Bailey, 1988; Kallio et al., 1994) by trapping O_2 and thereby shifts the metabolism pathway in favour of a higher ATP-producing terminal oxidase. Recently, it is claimed that tobacco transformed with VHb grows significantly faster, and germinates more easily than the wild type (Holmberg et al., 1997).

Intracellular expression of VHb in baker's yeast alters the aerobic metabolism of the yeast, probably by altering the mitochondrial electron transport pathway (Chen et al., 1994). Moreover, VHb could complement the deficiency of terminal oxidases in *E. coli*, suggesting that this protein can receive electrons during respiration and may transfer them to O_2 (Dikshit et al., 1992).

Cytoplasmic hemoglobins have also been proposed to function as metal chelators, mainly iron chelators; and to mediate oxidative phosphorylation (Wittenberg & Wittenberg, 1990). Obviously, more work is needed to elucidate the functions of these macromolecules in different organisms and under different conditions.

2.1.6. Hemoglobin gene expression and its regulation

As mentioned previously, human hemoglobin gene expression is stimulated by erythropoietin, which, in turn, is stimulated by oxygen deprivation. Thus, O_2 tension seems to play an important role in Hb gene expression in different organisms. *Vitreoscilla* Hb content increases almost 50-fold when oxygen pressure of the medium becomes limited (Wakabayashi et al., 1986). Non-symbiotic plant hemoglobins have been reported to occur in roots, cotyledons and aleurone layers (Taylor et al., 1994). Transformation of tobacco with *Parasponia* and *Trema* hemoglobin promoters indicates that the expression directed by the promoters is root tissue-specific (Bogusz et al., 1990). Studies on barley Hb gene expression indicate that this hemeprotein is significantly induced by O_2 shortage (Taylor et al. 1994).

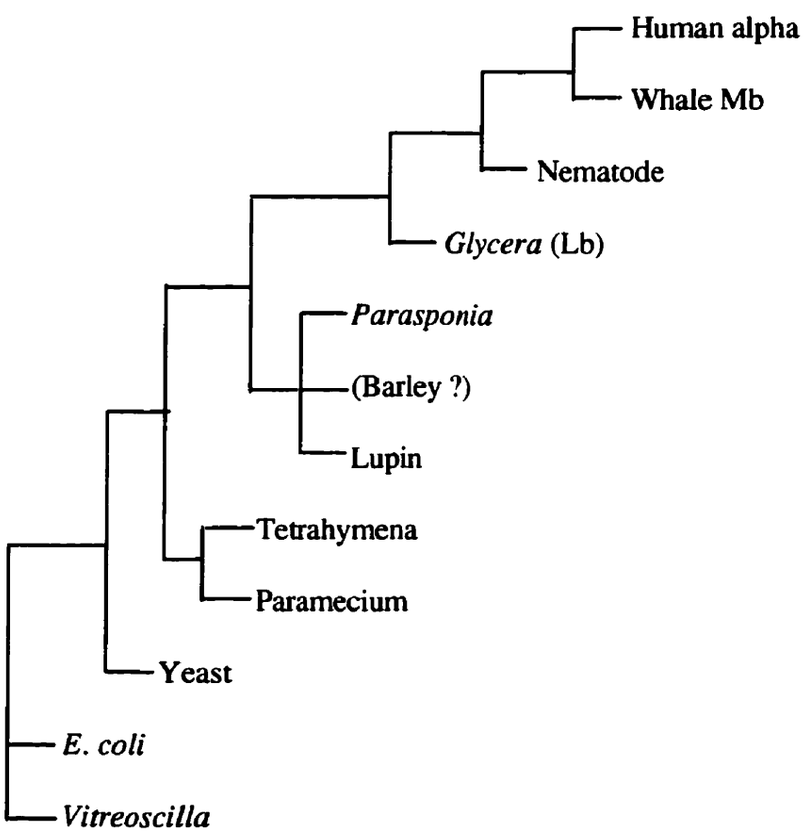
Like other legume genes, the leghemoglobin gene possesses specific nodulin motifs: 5'-AAAGAT-3' and 5'-CTCTT-3'. There are 6 to 7 nucleotides between the two motifs (Bogusz et al., 1990). These *cis*-elements direct leghemoglobin to be exclusively expressed in root nodules (Bogusz et al., 1990). The same *cis*-elements could be found in the promoter of *Casuarina* symbiotic Hb, indicating that all symbiotic hemoglobin genes possess a similar promoter region structure regardless of the origin.

2.1.7. Evolution of hemoglobin

Since the first non-animal source hemoglobin was purified from legume nodules about a half century ago, the origin and evolution of hemoglobin have attracted a lot of attention. Amino acid sequence analysis and quaternary structure analysis clearly show that the leghemoglobin, mammalian hemoglobin and myoglobin share a common origin (Hardison, 1996). It was once

widely accepted that the leghemoglobin had been horizontally transferred into the legumes from an animal source at the emerging stage of legumes (for reference, see Appleby, 1984). However, the recognition of hemoglobin occurrence in nodules from non-leguminous plants such as *Parasponia* and *Casuarina actinorhiza* discredited the hypothesis of a single event of horizontal gene transferral to a primitive legume. The recent findings of non-symbiotic hemoglobin in dicotyledonous plants, including the leguminous plant soybean, and monocotyledonous plants disproves the horizontal gene transmission hypothesis. Now, hemoglobins have been

Figure 1. Phylogenic tree of hemoglobin (from Zhu & Riggs, 1992; Barley Hb was not included in the original tree)



found and characterized in almost all types of organisms from prokaryotes including unicellular bacteria and cyanobacteria (blue-green algae) to eukaryotes ranging from fungi (yeast), monocotyledonous and dicotyledonous plants to almost all sorts of animals besides vertebrates. Zhu & Riggs (1992) analysed the phylogenies of hemoglobin and proposed the phylogenic tree shown in Figure 1. The time-scale of this phylogenic tree, from the divergence of the yeast and bacteria hemoglobin is at least 1.8 billion years (Appleby , 1992; Hardison, 1996).

Gene structure, especially the location of introns, could give us some information about the gene's evolution. Like other bacterial genes, bacterial hemoglobin genes do not possess any introns. Nor does the fungal hemoglobin gene. Interestingly, all animal hemoglobin genes either have two introns (vertebrate Hbs and annelid Hbs) or have no introns (insect Hbs); whereas both non-symbiotic and symbiotic plant Hbs have three introns. Most recently, barley Hb has been demonstrated to have three introns which are located at the identical positions as those found in soybean and *Parasponia* (Guy et al., 1997, GenBank, accession number: U94968). Further analysis indicates that all vertebrate and plant hemoglobins have almost the same intron structures, except that the middle intron is absent in the vertebrate Hb gene. These observations strongly suggest that the plant hemoglobin and animal hemoglobin originate from a common ancestral gene. It has been proposed that the common ancestor of plants and animals had a hemoglobin gene with three introns. This structure has been maintained in both symbiotic and non-symbiotic plant Hbs, whereas the central intron was lost during evolution in animals (Hardison, 1996).

Little is known about the functional evolution of hemoglobin. It is possible that the first ancient hemoglobin-like protein possessed a heme domain, which obviously had the capacity to bind O_2. As time went, this hemoglobin-like protein evolved into a more specific molecule to

function differently in different organism, from transporting O_2 in vertebrates to facilitating and buffering O_2 in vertebrates muscles and plant nodules, to accepting and transferring electrons to O_2 in bacteria.

2.2. Anaerobic responses in plants

With some exceptions, such as rice and barnyard grass which can survive in completely O_2-free root environments (Rumpho & Kennedy, 1981), most higher plants have an absolute requirement for oxygen for growth and development. The supply of O_2 to a tissue depends on its concentration and its diffusion rate in the surrounding medium. Under normal conditions, the supply of O_2 to the cells may not meet the requirement for respiration, resulting in hypoxia. Since the diffusion of oxygen is about 100-fold slower in water than in air (Thomson & Greenway, 1991), hypoxia is often found in tissues surrounded by a layer of water, such as roots and seeds in flooded soil. Thus, an occasional excess of water in the form of flooding can lead to a series of problems with plant growth and development.

Various responses have been observed in plants facing anaerobic stress, ranging from an alteration of metabolism to morphological changes. However, different plant species and even different tissues show different capacities of low oxygen stress tolerance. For example, some seeds can germinate in anoxia while others require full oxygen availability; some tissues can withstand days of hypoxia while others die in several hours (Crawford & Braendle, 1996). Despite the wide range of performances of plants subjected to hypoxia and anoxia, some common responses have been observed. Fermentative metabolism, including ethanol and lactate fermentation, is the most common, and also the best known response to oxygen deprivation.

Other responses such as *de novo* gene expression and protein synthesis have been widely demonstrated.

2.2.1. Anaerobic metabolism

Oxygen is the terminal electron acceptor in the mitochondrial electron transport process. Thus, the shortage of oxygen significantly affects mitochondrial respiration: pyridine nucleotides reduced in glycolysis and in TCA cycle cannot be reoxidized through the mitochondrial electron transfer chain. The accumulation of NADH results in a switch to a fermentative metabolism to allow NADH reoxidation and continuous ATP production through substrate phosphorylation, even though this ATP production is very low compared to that occurring during mitochondrial respiration.

Although several pathways have been proposed to reoxidize NADH in the absence of O_2 (Ricard et al., 1994; Kennedy et al., 1992), fermentative pathways leading to ethanol, lactate and alanine production are believed to be the major ones used to recycle pyridine nucleotides during anaerobiosis in plants (Ricard et al., 1994). All the fermentative end products (ethanol, lactate and alanine) are derived from pyruvate, the end product of glycolysis. Glycolysis is the pathway which is carried out in the cytoplasm and shared by both aerobic and anaerobic respiration.

Ethanol is the major product of fermentation in higher plant tissues. It is a consequence of the decarboxylation of pyruvate to acetaldehyde catalysed by pyruvate decarboxylase followed by the reduction of acetaldehyde which is driven by alcohol dehydrogenase. Lactate is produced from pyruvate by the action of lactate dehydrogenase. Carbons of alanine are derived from pyruvate while the nitrogen of alanine may come from diverse sources such as amides of asparagine and

glutamine which result from protein degradation during anaerobiosis (Menegus et al., 1989).

Lactate is often produced prior to ethanol in the first minutes after the transfer to hypoxia or anoxia (Roberts et al., 1984). Based on the observation of pH changes in cytoplasm due to increased lactate concentration, a hypothesis regarding the regulation of lactate and ethanol production under anaerobiosis has been proposed (Ricard et al., 1994). Under the normal O_2 tensions (normoxia), the cytoplasmic pH is relatively high, thus pyruvate decarboxylase (PDC) activity is low, resulting in low levels of substrate for alcohol dehydrogenase and low ethanol production. However, as O_2 availability is reduced, the fermentative pathway starts to work towards lactate production. Accumulated lactate acidifies the cytosol followed by the activation of PDC, subsequently, ethanol is produced by the action of alcohol dehydrogenase. Although this theory has wide support from various studies on anaerobic responses in different plant species, some exceptions have also been reported. For example, lactate remains low in some plants such as rice seedlings (Rivoal et al., 1991) or hypoxia-acclimated maize tips (Xia & Saglio, 1992), where ethanol production commences immediately upon hypoxia stress.

Other metabolites that are reported to accumulate under anaerobic conditions are succinate and malate (Ricard et al. 1994). Some contradictory reports regarding these anaerobic products have also been noted. For example, the level of malate in some cases does not increase, but slowly decreases under anoxia (Vanlerberghe et al., 1990). Succinate accumulation is quantitatively minor compared to lactate or ethanol production. In maize roots, succinate represents only 2.5% of the amount of lactate after 8 h of anoxia (Fan et al., 1988). In rice seedlings under anoxia, the rate of accumulation of succinate is less than 1% of that of ethanol (Rivoal et al., 1989).

Other possible pathways for NADH reoxidation in the absence of O_2 have been studied in plants. Nitrate has been proposed as a potential candidate for electron acceptor. Indeed, maize roots preincubated with nitrate had an increased level of nucleoside triphosphates, a lower amount of lactate and a better recovery after an anoxia treatment (Fan et al., 1988).

2.2.2 Molecular aspects of plants in responses to anaerobic stress

Anaerobic stress dramatically alters the profile of total protein synthesis in plants. Under anaerobic conditions, normal protein synthesis ceases. Around 20 polypeptides, referred to as anaerobic polypeptides (ANPs), account for more than 70% of total protein synthesis in maize roots under anoxia (Sachs et al., 1980). This selective synthesis of anaerobic proteins is the result of the selective translation of mRNA coding for the anaerobic proteins and the accumulation of anaerobic specific mRNA (Sachs et al., 1996). Most of the ANPs identified are found to be enzymes of glycolysis or sugar-phosphate metabolism, including sucrose synthase (Ricard et al., 1991), pyruvate decarboxylase (Laszlo & St Lawrence, 1983; Peschke & Sachs, 1994), aldolase (Kelley & Freeling, 1984a), enolase (Bailey-Serres et al., 1988), glucose-6-phosphate isomerase (Kelley & Freeling, 1984b), glyceraldehyde-3-phosphate dehydrogenase (Russell & Sachs, 1989), alcohol dehydrogenase (Sachs et al., 1980) and lactate dehydrogenase (Good & Paetkau, 1992).

The induction of alcohol dehydrogenase (ADH) by anaerobiosis is the most common response in plants. The ADH enzyme is a dimer. In maize, two *Adh* genes, *Adh1* and *Adh2*, are present, thus three isozymes, *i.e.*, ADH1.ADH1, ADH2.ADH2 and ADH1.ADH2, are found to be present. In barley, three *Adh* genes are present, leading to a total of five ADH isozymes in the cytoplasm of barley cells. As expected, the *Adh* gene is greatly induced by anoxia or hypoxia. In

maize roots, *Adh1* mRNA level is increased 50-fold by anaerobiosis (Gerlach *et al*, 1982). Similar results were observed in barley aleurone layers and roots (Hanson & Jacobsen, 1984), soybean seedlings (Russell et al., 1990), and rice seedlings (Kyozuka et al., 1994). Logically enough, ADH activity is also found to be significantly elevated by O_2 deprivation as well. The kinetics of induction of *Adh* mRNA shows that mRNA reaches its maximum level around 6 h of anaerobic conditions (Gerlach et al., 1982). In barley aleurone layers, ADH activity starts to increase within 6 h of anaerobiosis, and reaches its maximum level around 48 h (Hanson & Jacobsen, 1984; Taylor et al., 1994), suggesting ADH induction is rapid and also prolonged in plants in response to oxygen shortage.

Lactate dehydrogenase (E.C.1.1.1.27) is a tetramer of two randomly associated subunits (38 and 39 kD), (Rivoal et al., 1991). Two *Ldh* genes, *Ldh1* and *Ldh2*, have been isolated and analysed in various plant species. They encode 1.3 and 1.7 kilobase-pair transcripts, respectively. Like ADH, lactate dehydrogenase transcripts and activity are found to be elevated dramatically in barley aleurone tissue (Taylor et al., 1994; Hanson & Jacobson, 1984) and roots (Hoffman et al., 1986), maize roots (Christopher & Good, 1996), and rice seedlings (Rivoal et al., 1991). However, the half-life of LDH is decreased from 240 min in aerobic root extracts to 100 min in anaerobically induced root extracts, suggesting that the increased activity level of LDH is largely attributed to increased protein level, which correlates with increased transcript levels (Christopher & Good, 1996). In maize roots, the mRNAs of both *Ldh1* and *Ldh2* reach their peak at approximately 24 h of anaerobiosis, and thereafter, the transcripts decrease as hypoxia continues. LDH activity, however, continues to increase for at least 6 d of anaerobiosis (Christopher & Good, 1996). In barley aleurone layers, LDH activity reaches maximum levels around 48 h and

then remains at the same level until 72 h of anaerobiosis, the longest time tested in that experiment (Taylor et al., 1994).

Pyruvate decarboxylase (PDC), suggested to be one of the most important enzymes in anaerobic metabolism, catalyses the decarboxylation of pyruvate yielding CO_2 and acetaldehyde (precursor of ethanol). In maize, PDC activity increases 5 to 9- fold during anoxia, whereas PDC mRNA is induced 20-fold (Kelley, 1989). Three isozymes of PDC and their corresponding genes have been reported in maize. *Pdc1* and *Pdc3* mRNAs significantly increase within 6 h of anaerobiosis, and then remain at very high levels for prolonged time, whereas *Pdc2* transcripts do not show much induction in response to O_2 deprivation (Peschke & Sachs, 1994).

Some proteins that are not enzymes of glycolysis or fermentation are also induced by O_2 deprivation. For example, two anoxia-induced maize genes--*1005* and *1032*--that are not involved in glucose-phosphate metabolism (Sachs et al., 1996) are reported to be selectively synthesized. However, the function of these genes and the gene products are yet to be identified. The predicated amino acid sequence of *1005* has high homology to xyloglucan endotransglycosylase, a putative cell wall loosening enzyme that is proposed to play a role in wall metabolism during germination, cell expansion and fruit ripening (Sachs et al., 1996).

Hemoglobin is induced dramatically by O_2 deprivation in barley aleurone layers and roots, and maize roots (Taylor et al., 1994). In barley aleurone tissue, studies on Hb gene expression indicate that the gene is switched on very rapidly, and the transcription reaches its maximum around 6 h of anoxia. The gene responds to O_2 deprivation when O_2 tension is lowered to approximately 5-10%.

The expression of mitochondrial encoded polypeptides has been found to be affected by

anoxia. Mitochondria isolated from aerobic-treated and anoxia-treated rice seedlings show significant qualitative and quantitative differences in their polypeptides (Couee et al., 1992).

Regulation of ANPs has been studied in various plant species. Among the ANPs, alcohol dehydrogenase is the most thoroughly investigated. It has been established that *Adh* is induced by various stresses such as cold, heat-shock and the plant hormone ABA as well as O_2 deprivation (Dolferus et al., 1994; de Bruxelles et al., 1996). Promoter analysis by various techniques including footprinting and transgenic plant construction shows two types of DNA-binding factors associated with the promoter region. One is associated with the *cis*-regulatory anaerobic response element, whereas two additional factors bind only after *Adh1* has been induced by hypoxic stress (Paul & Ferl, 1991). Although both *Adh1* and *Adh2* respond to hypoxia, the signal transduction pathways for them appear to be different since *Adh1* and *Adh2* have different footprints under both aerobic and anaerobic conditions (Paul & Ferl, 1991). *Ldh1* and *Ldh2* may also be differently regulated in maize based on the induction kinetics observed (Christopher & Good, 1996). Some ANPs show different induction profiles to that of *Adh* and *Ldh*. The dramatic response of some genes to anaerobic stress is manifested more at the transcriptional level than at the protein level. For instance, the *Shrunken* (or *sh*) gene, encoding sucrose synthase (SS1), responds to anaerobic stress with a significant increase in mRNA levels (Springer et al., 1986) with no apparent increase in protein levels (McElfresh & Chourey, 1988), indicating that both transcriptional and translational regulation of ANPs occurs. An *In situ* hybridization study indicates that ANPs expressed in maize roots show different pattern and locations. For example, *Adh1* mRNA is induced in epidermis and cortex whereas *sh1* mRNA is induced greatly in the vascular cylinder, epidermis and pith (Rowland et al., 1989).

2.2.3. Anaerobiosis tolerance of plants

Tolerance of anaerobiosis, measured as the length of time a plant or plant tissue can be placed under anaerobic conditions and survive on return to air, varies greatly among species and tissues (Crawford & Braendle, 1996). Studies focused on the comparison of structural and metabolic patterns between tolerant and non-tolerant species and/or tissues have revealed a number of important points. Formation of aerenchyma in roots facing oxygen deprivation is a critical strategy for the roots or whole plants to survive under this stress. This structure has been observed in various plants ranging from monocotyledonous species such as rice, maize and barngrass to dicotyledonous plants like waxapple (Lin & Lin, 1992). It has been found that the aerenchyma formation in plant roots improves the oxygen supply to the root tissues (Drew et al., 1985). Although rice, maize and barley can develop aerenchyma, the capacity for aeration of the root system through aerenchyma development is ten times greater in rice than barley and four times than that found in maize (Perata & Alpi, 1993). However, in non-aerenchymatous plants like cucumber, O_2 transportation through leaves for root respiration in O_2-deficient incubation solution has been reported (Yoshida & Eguchi, 1994). Very similar results were also observed in non-aerenchymatous roots in *Pisum sativum L.* (Armstrong et al., 1982).

Metabolism alteration in plants subjected to O_2 shortage is another area which has drawn a lot of attention. The early assumption that ethanol, a major end product of the fermentative pathway, is toxic to plants, and therefore that flood-tolerance in plants depended on decreased ethanol production due to low ADH activity, has been questioned by recent studies in *Adh*-deficient mutants in maize (Kennedy et al., 1992). As expected, *Adh1*-null mutants lack ADH enzyme, thus have much less ability to produce ethanol. However, when such mutants are placed

under anaerobic stress together with the wild type plants, it is surprising that the mutants are more sensitive to O_2 deprivation than that of the wild type. Thus, ADH appears to be an important and necessary feature for plants to survive when O_2 deficit occurs. The physiological role of induced alcohol dehydrogenase in flooding-tolerance has yet to be determined.

It has been proposed that cytoplasmic acidosis may be a cause for anoxia intolerance (Roberts et al., 1984). In *ADH1*-null mutant, no alcoholic fermentation occurs with the continuous fall in pH under anoxia. Indeed, plant species exhibiting greater anoxia tolerance (such as *Echinochloa sp.* and rice) undergo little or no cytoplasmic acidosis during anoxic treatment (Kennedy et al., 1992). Low oxygen acclimated maize seedlings show significant anoxia-tolerance over non-acclimated seedlings with pH-regulation and less cytoplasmic acidosis (Xia & Saglio, 1992; Xia & Roberts, 1994). Moreover, the predominant fermentative end product retained in the cells of acclimated maize root tips is alanine rather than ethanol or lactate (Xia & Roberts, 1994).

Post-anoxic injury, the damage taken place when tissues that have been exposed to anaerobiosis are restored to air, is one problem that significantly affects the survival of plants or plant tissues after the stress. Post-anoxic oxidation of reduced iron triggers off the generation of active oxygen species (superoxide *etc.*), followed by H_2O_2 through the action of superoxide dismutase (SOD). H_2O_2 can then react with ethanol that accumulated under anoxia to generate acetaldehyde (Crawford & Braendle, 1996) as shown.

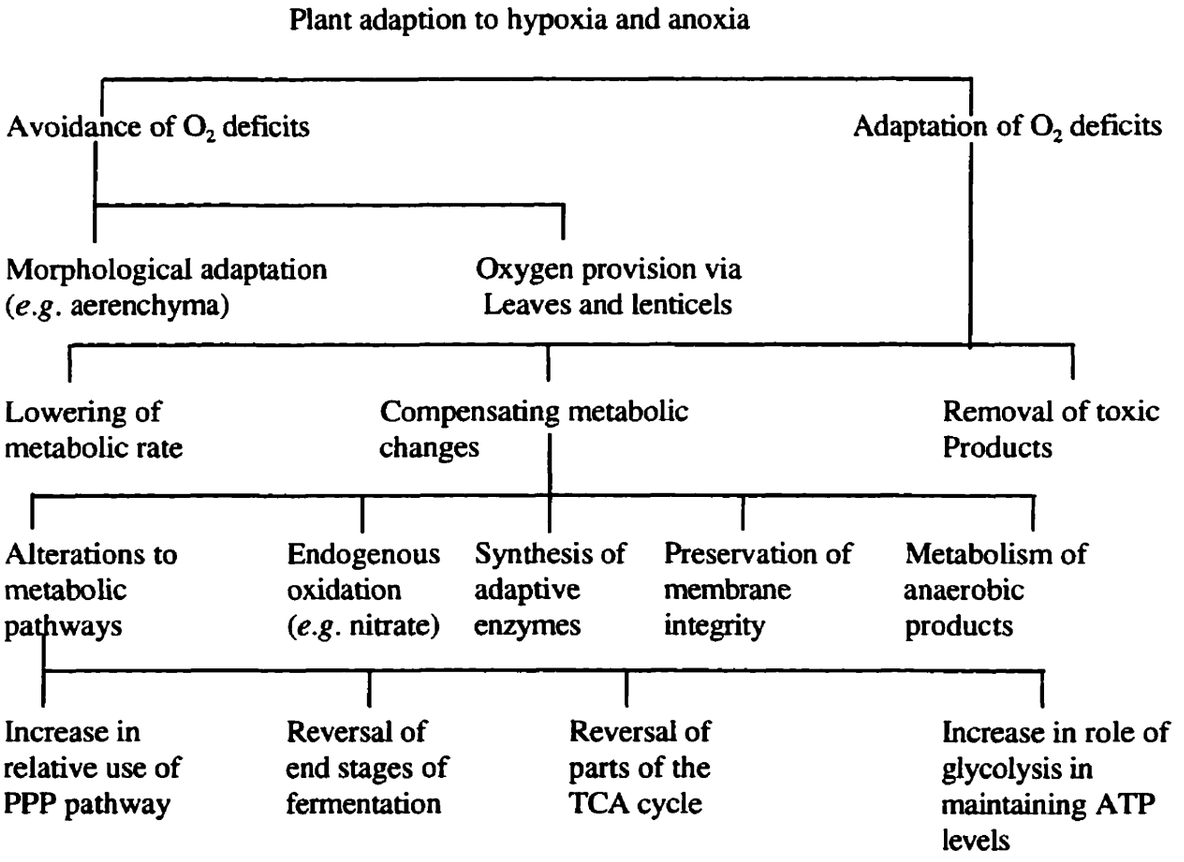
$$Fe^{2+} + O_2 \text{ ---> } Fe^{3+} + O_2^{\cdot-}$$

$$O_2^{\cdot-} + O_2 \text{ ---> SOD---> } H_2O_2$$

$$H_2O_2 + C_2H_5OH \text{ ------> } CH_3CHO + 2H_2O$$

Thus, accumulated ethanol is potentially threatening to the cell membranes due to its rapid oxidation to acetaldehyde on re-exposure to air. Anaerobic stress tolerant species have been found to have higher defence capacities by possessing increased levels of SOD and other anti-oxidants such as ascorbic acid, α-tocopherol and glutathione (Crawford & Braendle, 1996).

Figure 2. Plant adaptation to hypoxia and anoxia (from Crawford & Braendle, 1996)



Barley is a flooding sensitive species (Hoffmann et al., 1986). Barley seeds cannot germinate under anoxia, and the adult plant may die within a very short time under anaerobic conditions. However, barley aleurone layers are highly-tolerant to anoxia, and can survive prolonged O_2 deprivation (Hanson & Jacobsen, 1984). Alcohol dehydrogenase and lactate dehydrogenase have been reported to be significantly induced by O_2 shortage (Hanson & Jacobsen, 1984; Taylor et al., 1994). The mechanism of the tolerance to anaerobiosis in this tissue is not yet fully understood.

In conclusion, metabolism and morphological alteration occurs when plants face anaerobic stress. As shown in the scheme (Fig. 2) proposed by Crawford & Braendle (1996), different mechanisms of survival may be used when anaerobiosis happens. Thus, plants vary in their ability of tolerance to the stress.

2.3. Mitochondrial Respiration

The mitochondrion is the site of bio-oxidation and energy production in the form of adenosine triphosphate (ATP). The respiration carried out in the mitochondria plays a critical role in maintaining cell viability. Thus, vigorous tissues usually have a high respiration rate, whereas matured tissues possess a relatively low respiration rate.

Mitochondrial respiration is virtually a complete oxidation of metabolites, *e.g.* glucose, to carbon dioxide and water as the final products accompanied by ATP production (Taiz & Zeiger, 1991). During this process, electrons are transferred to oxygen by a series of electron transport

components in the inner membrane of the mitochondrion. The overall process from glucose to CO_2 could be described by the following equations:

$C_6H_{12}O_6 + 6\ H_2O \text{ ---> } 6\ CO_2 + 24H^+ + 24e^-$ (1)

$6O_2 + 24H^+ + 24e^- \text{ ----> } 12\ H_2O$ (2)

In the first step of the process, the glucose carbons are oxidized, whereas in the second step of the reaction, molecular oxygen is reduced by accepting electrons generated from the first step.

In this part of the review, I will focus on the electron transport and the transport-associated oxidative phosphorylation in plant mitochondria.

2.3.1. Plant mitochondrial electron transport and oxidative phosphorylation

Respiratory electron transport is carried out in the inner membrane of the mitochondrion. The inner membrane has a high protein content (over 70% on a dry weight basis) and a large range of polypeptide types, accounting for 30% of the total organelle protein content (for reference, see Douce, 1985). Several membrane-bound redox components, such as flavoproteins, iron-sulfur proteins, ubiquinone and cytochromes that catalyze the reduction of O_2 by the reduced coenzymes ($NADH_2$ and $FADH_2$) generated intramitochondrially are attached on the inner membrane. These components are arranged in a sequence according to their redox potentials (Siedow & Umbach, 1995). Like electron transport chains in mammalian mitochondria, the plant mitochondrial respiratory chain has five protein complexes, designated complex I, complex II, complex III, cytochrome *c* and complex IV or cytochrome *c* oxidase.

Complex I (NADH-Ubiquinone-oxidoreductase) is the entry point for the redox

equivalents of NADH produced in the matrix space during the course of substrate oxidation. Complex I contains an non-covalently bound flavin mononucleotide (FMN), several iron-sulfur centres and at least two molecules of ubiquinone, reaching a molecular weight of 670 kD (Hatefi & Galante, 1977). Complex I functions as the first coupling site, carrying reversible electron flux from NADH to quinone coupled to generation of proton gradient ($\Delta\mu H^+$).

Complex II (succinate-ubiquinone-oxidoreductase) is the smallest unit with a molecular weight around 125 kD. It contains the dimeric citric acid cycle enzyme, succinate dehydrogenase and three other small hydrophobic subunits (Douce, 1985). This complex catalyses the oxidation of succinate to fumarate during the operation of the TCA cycle, passing the resulting electrons to ubiquinone (Siedow & Umbach, 1995). The standard redox potential for electron transfer from succinate to ubiquinone is insufficient to provide free energy (ΔG) to drive ATP synthesis (Douce, 1985).

Complex III (ubiquinone-cytochrome *c* oxidoreductase) is the site of the respiratory chain responsible for electron transfer from ubiquinol (QH_2) to cytochrome *c* (Douce, 1985). It contains two *b*-type cytochromes (b_{562}, b_{566}), one cytochrome c_1 and one molecule of ubiquinone and one iron-sulfur cluster (2Fe-2S). Complex III functions as the second coupling site, carrying reversible electron flux from ubiquinone to cytochrome c associated to the generation of $\Delta\mu H^+$.

Cytochrome *c* is loosely bound to the outer surface (*p*-side) of the inner membrane of mitochondria (Siedow & Umbach, 1995). It alternatively binds to cytochrome c_1 of complex III and to complex IV and thus functions to shuttle electrons between them (Douce, 1985). Cytochrome *c* is a small (12.5 kD) peripheral protein and is the only protein of the electron transfer chain that is not part of an integral membrane protein complex.

Complex IV (cytochrome *c* oxidase) is the terminal complex of the respiratory chain, composed of at least seven subunits (Douce, 1985). Subunit I and II contain all four of its redox-active centres: two *a*-type cytochromes (*a*, a_3) and two Cu atoms. Cytochrome *a* and Cu atom (Cu_A.*a*) are of low potential (approx. 0.24 V), whereas cytochrome a_3 and Cu atom (Cu_B.a_3) are of higher potential (approx. 0.34) (Douce, 1985). Cytochrome *a* reacts with cytochrome *c*, whereas cytochrome a_3 reacts with O_2. The reaction carried out by the complex is a one-electron oxidation of four consecutive reduced cytochrome *c* molecules and the concomitant four-electron reduction of one O_2 molecule:

4 cytochrome c^{2+} + 4 H^+ +O_2 ---> 4 cytochrome c^{3+} +2 H_2O

Complex IV also functions as the third coupling site through the above reaction coupled to the generation of $\Delta\mu H^+$.

Besides the main electron transport pathway from NADH to cytochrome *c* oxidase, another pathway has been found in plant mitochondria. In this pathway, electrons are directly passed to oxygen from ubiquinone by the action of an alternative oxidase (Douce, 1985; McIntosh, 1994). Since electrons are transmitted to O_2 without the participation of complex III and complex IV in this pathway, less coupling of $\Delta\mu H^+$ occurs. Although significant progress, including gene cloning, has been made recently (Vanlerberghe & McIntosh, 1996), the physiological significance of the alternative oxidative pathway is not yet fully understood.

Electron transport from reduced *CoA* to oxygen through the respiration chain is coupled to ATP production from ADP and P_i in the mitochondria. The synthesis of ATP is catalysed by ATP synthase (or F_0F_1-ATP synthase) accompanied by proton re-entry to the mitochondria via this complex. ATP synthase is not part of the electron transfer chain *per se*, but its association

with inner membrane and its primary role in oxidative phosphorylation have led to its being referred to as complex V (Hatefi, 1985). The complex has a molecular weight of 500 kD, containing two empirically defined components F_1 and F_0. F_0 is a water-insoluble transmembrane protein composed of four or five types of subunits that contain a channel for proton translocation. F_1 is a water-soluble peripheral membrane protein composed of five types of subunits. When bound to the inner mitochondrial membrane, the F_0F_1-ATP synthase complex catalyses the dehydration of ADP plus P*i*, leading to ATP synthesis (Douce & Neuburger, 1989).

Since three complexes in the electron transport chain are coupled to $\Delta\mu H^+$ generation, oxidation of one molecule of NADH through the mitochondrial respiratory chain to O_2 results in the synthesis of three molecules of ATP. The alternative pathway, however, only leads to one ATP.

2.3.2. Respiratory inhibitors

Numerous respiratory inhibitors have been found. Most of these inhibitors act either by binding to a specific complex in the respiratory chain or by interfering with ATP production in the mitochondria.

Antimycin A , an antibiotic, is a specific inhibitor of the cytochrome *c* reductase (complex III). It binds to cytochrome *b*-*c*1 complex and thus blocks the electron transport from ubiquinone to cytochrome *c*.

Like hemoglobin, cytochrome a_3 of cytochrome *c* oxidase can bind O_2 as well as other exogenous ligands such as CN^-, N_3^-, CO and H_2S. Thus these compounds are strong and specific inhibitors of the terminal oxidase, blocking electron transport to O_2 from cytochrome *c*. The

inhibition of cytochrome *c* oxidase by CO can be released by light. For instance, electron transport in the freshly cut disks of potato tissue is inhibited by CO, however, the inhibition is fully reversed by light (Thimann et al., 1954)

Alternative oxidase may function as a pathway for electron transport to O_2 when cytochrome *c* oxidase pathway is saturated or blocked (McIntosh, 1994). This pathway is featured by its insensitivity to CN^-, CO or other complex IV inhibitors (Bonner, 1964) and antimycin A (Vanlerberghe & McIntosh, 1996). Several inhibitors of alternative oxidase have been widely used (Siedow & Umbach, 1995). The most common one is hydroxamic acid and its derivatives such as benzhydroxamic acid (BHAM) and salicylhydroxamic acid (SHAM). *n*-Propylgallate and 8-hydroxyquinolin are also widely used (McIntosh, 1994). All of these compounds are ferric ion chelators, suggesting that iron may be a component of the enzyme (Douce & Neuburger, 1989). Indeed, in the presence of the Fe^{2+} chelator *o*-phenanthroline, induction of the alternative oxidase in *H. anomala* by addition of inhibitors of mitochondrial protein synthesis leads to the appearance of the 36-kD alternative oxidase protein but no alternative pathway activity (Minagawa et al., 1990). However, upon addition of Fe^{2+} to the medium, alternative pathway activity appears rapidly.

Many chemicals, including 2,4-dinitrophenol (DNP) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), are found to uncouple electron transport and oxidative phosphorylation in mitochondria. DNP and FCCP are lipophilic weak acids that therefore readily pass through membranes, and thus increase the rate of H^+ leakage and eventually equilibrate the electrochemical H^+ gradient across the mitochondrial inner membrane. Uncouplers release respiratory control completely and suppress all energy-linked reaction including ATP production

(Douce, 1985). Addition of an uncoupler to the incubation medium dramatically increases O_2 uptake rates in cells or tissues.

Oligomycin, an antibiotic produced in *Streptomyces diastatochromogenes*, is a strong inhibitor of ATP synthase in mitochondria. It binds to a subunit of F_0 and therefore interferes with H^+ transport through F_0, resulting in blockage of oxidative phosphorylation. Oligomycin has been reported not to affect plant mitochondrial state 4 rates, the respiration rates in the absence of ADP, while it completely inhibits the increment of respiration due to added ADP (Ikuma & Bonner, 1967).

Respiratory inhibitors have been widely used to study various physiological processes and the involvement of possible respiratory pathways or energy metabolism ranging from seed germination to ion uptake in roots to photosynthesis in leaves. For instance, cyanide, carbon monoxide, hydrogen sulphide and 2,4-dinitrophenol (DNP) are reported to effectively break seed dormancy in rice (Roberts, 1964) and other cereal seeds (Major & Roberts, 1968). By various tests with respiratory inhibitors, a conclusion regarding the seed dormancy and respiratory pathways has been proposed by Roberts and Smith (Roberts & Smith, 1977). They proposed that the pentose phosphate pathway (PPP) plays an important role in seed germination. Respiratory inhibitors including CO, CN^- and H_2S can effectively shift the glycolysis-TCA pathway to PPP by blocking terminal oxidase activity and therefore reduce consumption of limited oxygen supply and make it available for NADPH reoxidation (Roberts & Smith, 1977).

Plant roots selectively absorb ions from their environments. Interfering with energy production by incubating barley seedling roots with oligomycin significantly affects ion absorption (Jacoby & Plessner, 1970). More interestingly, oligomycin can affect barley leaf protoplast

photosynthesis by perturbing oxidative phosphorylation (Kromer et al., 1993). Very similar results are observed in winter rye leaves (Hurry et al., 1995).

2.3.3. Energy metabolism in plants

Energy production and utilization are highly controlled and precisely regulated. For vigorous cells or tissues, where metabolism is actively carried out, energy metabolism is matched to the requirement of ATP. Electron flux from reduced CoA to oxygen through the cytochrome pathway is coupled to the production of 3 ATP. This process is ADP and P_i dependent. In the absence of ADP, oxygen consumption by mitochondria is low. However, when ADP is present in excess, O_2 uptake by mitochondria is greatly induced (Douce & Neuburger, 1989). The former is referred as "state 4", while the latter is defined as "state 3". Under physiological conditions, the ATP/ADP ratio is suggested to be critical for mitochondrial respiratory control and ATP production. When the ratio is sufficiently high, respiration is reduced; whereas when the ratio is low, respiration is enhanced. Under some circumstances, electrons may pass from ubiquinone to oxygen through the alternative oxidase, which is not coupled to ATP production. The availability of O_2 in the medium, of course, plays a significant role in ATP production (for reference, see Douce, 1985).

Energy charge, a parameter indicating energy state in cells or tissues, is widely used. Under physiological conditions, the energy charge is about 0.8 to 0.9 (Pradet & Raymond, 1983). However, under anoxia or hypoxia, ATP production through oxidative phosphorylation is limited due to insufficient oxygen, and energy charge could significantly drop (Saglio et al., 1980; Pradet & Raymond, 1983). Subsequently, glycolysis and the fermentative pathways are activated

because of the Pasteur effect, which can slightly increase the energy charge due to substrate phosphorylation. However, ATP production from fermentation is significantly lower than that from mitochondrial respiration. Complete oxidation of a glucose to CO_2 from glycolysis-TCA matched to the cytochrome pathway produces 38 ATP, whereas one glucose oxidation to ethanol or lactate through glycolysis-ethanol or lactate fermentative pathway only generates 2 ATP (Douce, 1985).

Ethanol/or lactate fermentation:

$C_6H_{12}O_6 + 2ADP + 2\ Pi \text{ ----> } 2\ Lactate\ (or\ Ethanol) + 2H^+ + 2ATP$

Aerobic metabolism of glucose:

$C_6H_{12}O_6 + 38ADP + 38Pi + 6O_2 \text{ ---> } 6CO_2 + 44H_2O + 38ATP$

For most fast growing tissues in plants, *e.g.*, root tips, high ATP utilization decreases the ATP/ADP ratio and energy charge (Douce, 1985), which in turn can increase respiration in order to match the energy requirement.

2.4. Signal transduction in plants

Responses to developmental and environmental cues in higher organisms occur by stimulus-response coupling: a stimulus is perceived by the cell, a signal is generated and transmitted (signal transduction), and a biochemical change occurs (the response) (Bowler & Chua, 1994). This process most often requires the recognition of the stimulus by a receptor and the subsequent use of chemical second messengers, such as calcium and cyclic AMP, and/or effector proteins to transmit a signal that will then trigger the appropriate response. In simple

terms, the sequence of response for a specific stimulus in plants is: signal recognition-signal transduction-responses.

Signal recognition as the first step in various responses is yet to be demonstrated. Very limited data are available regarding the receptors that accept specific stimuli, even though many attempts have been reported (Bowler & Chua, 1994). The best known signal receptor in plant systems is phytochrome (Furuya, 1993). Although some data positively indicate the existence of plant hormone receptors, they have yet to be unequivocally identified.

Much progress has been made in signal transduction in recent years. Ca^{2+} and Ca^{2+}-dependent calmodulin, cAMP and GTP-binding proteins have been proved to be involved in numerous signal transduction processes. The cascades of protein phosphorylation/ dephosphorylation, which are catalysed by protein kinases and protein phosphatases, are believed to be major events of signal transduction. To date, however, not even a single complete pathway has been demonstrated.

De novo protein production or specific gene induction is one of the typical and fast responses to a stimulus (Deng, 1994). Thus, analysis of gene structure, especially the elements in the putative promoter region, often results in the discovery of conservative motifs which are critical to direct the response of the gene to a signal.

In this section of review, I will focus on the possible components of signal transmission chain in plants, and discuss how a specific gene can receive the signal.

2.4.1. Ca^{2+}/CaM in signal transduction

Cytosolic calcium has been suggested to be a second messenger for decades. Indeed, Ca^{2+}

is involved in many events in plants subjected to either developmental cues or environmental stimuli (Trewavas & Knight, 1994). For instance, Ca^{2+} plays a role in the phytochrome-mediated process in plants (Wei & Deng, 1996). Red/far-red light reversibly leads to transient changes in plant cells, which precedes the physiological responses to the light stimulus (Shacklock *et al*, 1992). Microinjection of Ca^{2+} and Ca^{2+}-activated calmodulin (Ca^{2+}/CaM) into phytochrome-deficient tomato cells results in stimulation of chloroplast development in these cells (Neuhaus et al., 1993).

It is well known that metabolic and morphological alteration occurs when plants are subjected to unfavourable conditions, such as drought, temperature extremes and oxygen shortage. Cytosolic Ca^{2+} is one of the earliest events during the process. Moreover, control of Ca^{2+} movement by adding the divalent ionophore A23187 (Reed & Lardy, 1972) and the Ca^{2+} chelator EGTA significantly affects the responses. For example, alcohol dehydrogenase induction by anoxia or hypoxia can be selectively blocked by the Ca^{2+} channel blocker, ruthenium red, in maize roots (Subbaiah et al., 1994a). Using fluorescence imaging and photometry of Ca^{2+} in maize suspension cultured cells, Subbaiah *et al.* (1994b) found that oxygen deprivation causes an immediate increase in Ca^{2+} influx ($[Ca^{2+}]_i$) and this is reversible within a few seconds of reoxygenation. These findings support the view that $[Ca^{2+}]_i$ is a physiological transducer of anoxia in plants.

ABA can induce stomatal closure in various plants (Ward et al., 1995). One of the earliest known responses of guard cells to ABA is an increase in cytosolic Ca^{2+} (McAinsh et al., 1990), such an increase is sufficient to induce stomatal closure (Gilroy et al., 1990).

Mechanical stimuli such as wind and touch and their effects on plants have been a subject

of interest for more than one century (Trewavas & Knight, 1994). Ca^{2+} is shown to mediate the responses in plants to mechanical stimuli. By establishing transgenic tobacco with a calcium-sensitive luminescent protein, aequorin, from the jelly fish *Aequorea victoria*, direct observation of Ca^{2+} movement, accumulation and distribution has been carried out (Knight et al., 1992). Wind causes immediate increases in cytosolic Ca^{2+}, which is proposed to be responsible for the wind-induced movement of tissues (Knight et al., 1992). Touch as well as cold-shock and elicitors have a similar effect on Ca^{2+} in the cytoplasm of transgenic tobacco cells (Knight et al.,1991). Moreover, EGTA and calmodulin-binding inhibitors can negate rub-induced growth reductions in soybean (Jones & Mitchell, 1989). Touch stimulation of *Arabidopsis* massively induces the expression of five touch genes, three of which have been identified as CaM or CaM related proteins (Braam & Davies, 1990).

Microbial invasion results in a series of responses including hyposensitive and metabolic reactions in plants. Elicitors have very similar effects and can lead to a response in plants identical to what a pathogen induces. Thus, elicitors are often used to study the influence of microbes on plants. Ca^{2+} changes in the cytoplasm are among the earliest occurring events when plants or plant tissues are inoculated with the elicitors (Knight et al., 1991). Like other Ca^{2+} mediated responses, calcium chelators such as EGTA can effectively abolish the effects of elicitors (Knight et al., 1991).

Although it has been suggested that Ca^{2+} influx from the extracellular space is required for stomatal closing (DeSilva et al., 1985), recent work indicates that Ca^{2+} is released from intracellular sources (Knight et al., 1991; 1992; Trewavas & Knight, 1994; Allen et al., 1995). Ruthenium red, a dye, is believed to inhibit release of Ca^{2+} from the rough ER (Denton et al.,

1980). It is generally assumed that ruthenium red modifies Ca^{2+} release from internal stores (Trewavas & Knight, 1994). Indeed, in cultured maize suspension cells, anoxia-induced increase in [$Ca^{2+}]_i$ in maize cells occurs as a single peak without any dual components and is independent of extracellular Ca^{2+} (Subbaiah et al., 1994b).

Ca^{2+}-independent responses have been reported in various plants or plant tissues subjected to a stimulus (Ward et al., 1995). In guard cells, ABA-induced signalling appears to proceed through parallel Ca^{2+}-dependent and Ca^{2+}-independent pathways, both of which may be sufficient to cause stomatal closure (Allan et al., 1994). In barley aleurone layers, the phytohormone gibberellic acid (GA) increases Ca^{2+} and CaM whereas abscisic acid (ABA) induces changes in Ca^{2+} and CaM. Microinjection of caged Ca^{2+} or CaM failed to mimic GA action on α-amylase gene expression and ABA action on early-methionine-labelled-protein (*Em*) gene transcriptions (Gilroy, 1996), suggesting that GA and ABA signals are transmitted by Ca^{2+}- and CaM dependent and Ca^{2+}- and CaM-independent systems in barley aleurone cells (Gilroy, 1996).

2.4.2. Role of reversible protein phosphorylation in signal transduction in plants

Reversible phosphorylation of proteins controls many cellular processes in plants and animals (Hunter, 1995). The phosphorylation status of proteins is regulated by the activities of protein kinases and protein phosphatases. Phosphorylation of eukaryotic proteins occurs predominantly (97%) on serine and threonine residues and to a lesser extent on tyrosine residues (Smith & Walker, 1996). In plant cells, accumulating evidence indicates that protein phosphorylation / dephosphorylation is modulated in response to different external signals

including light, gravity, hormones and stress factors. An increasing number of plant protein kinases and phosphatases have been reported in recent years. As in animals, a number of kinases and phosphatases respond directly to secondary messengers such as Ca^{2+} in plants. Thus, a specific signal transduction pathways with the involvement of protein phosphorylation / dephosphorylation can be blocked by elimination of a specific protein kinase or phosphatase or by inhibiting its activity.

A mutagenic approach has been widely used in the signal transduction studies (Bowler & Chua, 1994). The well-known ABA-insensitive mutants (*ABI*) of *Arabidopsis* are severely impaired in a wide spectrum of ABA responses, including excessive water loss and reduced seed dormancy. One such mutant gene, *abi1*, has recently been cloned (Leung et al., 1994). The gene product, ABI1, has a novel structure containing a domain highly homologous to the serine/threonine protein phosphatase type 2C, together with an N-terminal Ca^{2+} binding site. Although it is not clear whether phosphorylation or dephosphorylation is the forward reaction for the *ABI1* regulated step in ABA responses (Bowler & Chua, 1994), phosphorylation/dephosphorylation of protein plays a central role in this process.

Ethylene response mutants have been isolated in *Arabidopsis* by using a genetic approach, defining several *loci* involved in ethylene signalling (Roman & Ecker, 1995). The ETR1 gene, which corresponds to one of these *loci*, has been cloned. The gene product of ETR1 is a putative transmembrane histidine protein kinase similar to bacteria two-component regulators (Chang et al., 1993). The N terminus may function as an ethylene-receptor, whereas the C-terminal region may act as a signal transmitter via auto-phosphorylation of histidine (Bowler & Chua, 1994). Two additional genes, CTR1 and HLS1, that act downstream of ETR1 in ethylene signalling have been

cloned (Kieber et al., 1993; Lehman et al., 1996). CTR1 is a *Raf*-like protein kinase and negatively regulates ethylene responses (Kieber et al., 1993). A *LAMMER* family kinase, PK12, has been identified recently in *Arabidopsis* (Sessa et al., 1996). This kinase is suggested to be involved in ethylene signal transduction since (1) it is greatly induced by ethylene; and (2) it shows similar activation characteristics as ETR1 and CTR1. Overall, in simple terms, ethylene signal transduction requires the involvement of reversible protein phosphorylation.

Rhizobium meliloti FixL and FixJ have been shown to play a crucial role in the induction of nitrogen fixation genes in nodules (Gells-Gonzalez et al., 1991). FixL is a kinase and hemeprotein with the characteristic oxy and deoxy spectra of Hb (Gilles-Gonzalez et al., 1991; 1993). Under anaerobic conditions, deoxy-FixL transfers a phosphoryl group to the transcriptional activator FixJ, which, in turn, enhances FixJ's activity and induces the nitrogen fixation genes. This system indicates that reversible protein phosphorylation is crucial for the oxygen deprivation signal transmission to the target genes, even for the relatively simple two component systems.

Additional evidence that protein phosphatases and kinases are involved in plant signalling pathways comes from numerous kinase and phosphatase inhibitor experiments. For instance, okadaic acid, an inhibitor of protein phosphatase type 1 and type 2A, completely blocks every aspect of the GA response measured by Kuo et al. (Kuo et al., 1996) in barley aleurone layers, strongly suggesting that okadaic acid-sensitive phosphatases plays a crucial role in the GA signal transduction pathway. Similarly, by using protein phosphatase inhibitors, a number of processes, including light, pathogen and plant hormone mediated responses, that require protein phosphorylation / dephosphorylation have been identified (Smith & Walker, 1996).

2.4. 3. G-protein and signal transduction in plants

Heterotrimeric GTP-binding proteins (G-proteins) are essential components of many signal transduction pathways in animal cells (Birnbaumer et al., 1990). G-proteins are characterized by highly conserved structure, related molecular mechanisms and association with homologous membrane-spaning receptors (Legendre et al., 1992). A common feature of these proteins is that a receptor-mediated stimulus induces the exchange of GTP for protein-bound GDP. The active protein (GTP-bound) then interacts with an effector molecule, *e.g.*, a phospholipase, cyclase, ion channel, phosphodiesterase *etc.*, to trigger a cascade of amplification and transmission of the signal to downstream targets. An endogenous GTPase activity hydrolyzes the GTP to GDP , thus switching off the process. Cholera toxin can modify the G-protein by ADP-ribosylating a subunit of the G-protein and thus inhibits or reduces the GTPase activity of the protein. In contrast, pertussis toxin catalyses ADP-ribosylation of the inhibitory Gα subfamily Gαi and therefore preventing the activation of the protein (Gilman, 1987).

G-proteins have been found in different plant sources (Romero & Lam, 1993). A cDNA encoding a putative Gα protein has been cloned from *Arabidopsis* (Ma *et al*, 1990). However, most evidence regarding the involvement of G-protein in signal transduction in plants is based on the specific modulators of G-protein experiments. For instance, mastoparan and cholera toxin are used to study elicitor induced rapid oxidative burst in soybean cells (Legendre *et al*, 1992). Mastoparan, a specific activator of G-proteins, induces an oxidative burst in the absence of elicitor stimulation, thus mimicking an activated receptor as it is thought to do in mammalian systems (Legendre et al., 1992). Similarly, cholera toxin and pertussis toxin can mimic light to activate expression of genes encoding the major chlorophyll *a*/*b*-binding protein (s) (CAB) in

phytochrome-mediated *cab* gene activation (Romero & Lam, 1993). These data, together with the observation of Ca^{2+}/CaM in *cab* gene induction, lead to the model that activation of GTP-binding proteins is an early step in P_{fr} (phytochrome far-red form) signal transduction, whereas Ca/CaM and cGMP act later, on separate pathways (Terzaghi & Cashmore, 1996).

In summary, the signal transduction in plants is a very complicated process involving numerous reactions and different components. Ca^{++}, reversible protein phosphorylation and G-proteins are apparently involved in the process.

3. Materials and Methods

3.1. Plant materials

3.1.1. Barley aleurone layers

Seeds of barley (*Hordeum vulgare* L. cv Harrington, provided by Canadian Grain Commission, Winnipeg, Manitoba) were dehusked in 50% H_2SO_4 for 2 h followed with thorough rinsing in tap-water. The seeds were air-dried at room temperature and stored at 4°C.

The dehusked seeds were deembryonated and a small portion of the distal end was removed. The resulting half seeds were surface sterilized for 30 min in 1% (w/v) NaOCl (Javex) and rinsed thoroughly in distilled water. After 2 days of imbibition at 22°C in darkness, the aleurone layers were separated from the starchy endosperm. Twenty-five layers were placed in a sterile 50 mL conical flask containing 1.5 mL incubation medium (either double-distilled water or 0.1 M phosphate buffer, pH 7.0) and incubated with slow agitation (65 cycles/min) at room temperature.

3.1.2. Barley coleoptiles

Barley seeds (cv. Harrington) were surface sterilized in 1% (w/v) NaOCl (Javex) for 30 min at room temperature. After rinsing in tap-water for up to 8 times with total 15 min, the seeds were placed on a sterilized filter paper set in the germinating box (13.5 x 18 cm). After incubating in darkness for 2-3 days, when the coleoptiles were about 2 cm in length, the seedlings were treated according to the purpose of the experiment.

3.1.3. Barley seedlings

Untreated barley seeds (cv. Harrington for Northern blot analysis; cv. Bananza and Himalaya for Southern blot analysis) were planted in pots of soil, and grown in the growth chamber (27°C-20°C, 16 h light/ 8 h dark). Two to three weeks later, when the seedlings were about 2- to 3-leaf stage, the plants were treated as described below. Roots and/or leaves were subsequently harvested.

3.1.4. Rice plants

Rice (*Oryza sativa L.*) seeds were soaked in tap-water for 2 days at 30°C. After the water was poured off, the seeds were kept in a beaker with 100% moisture and incubated at 30°C for 2-3 days until the seeds germinated. The seeds were then planted in pots of soil, and placed in a container with a sufficient amount of water, and grown in the green house (approx. 30°C-25°C) until 5-leaf stage. The leaves were then harvested and used to isolate genomic DNA.

3.1.5. Other plant materials

Maize (*Zea mays L.*), oat (*Avena sativa L.*), wheat (*Triticum aestivum L.*), rye (*Secale cereale L.*), bean (*Ricinus communis L.*) and rapeseed (*Brassica napus L.*) seedlings were grown in an identical manner as the barley seedlings. Young leaves were harvested and used to extract genomic DNA.

3. 2. Inhibitor experiments

3.2.1. Respiratory inhibitors

Unless otherwise stated, freshly prepared barley aleurone layers were incubated in 0.1 M

phosphate buffer (pH 7.0) containing various additions. The cytochrome *c* oxidase inhibitor KCN was used at a concentration of 0.8 mM. The cytochrome *c* reductase inhibitor antimycin A (diluted from 27 mM stock in 2-propanol) was used at a concentration of 0.2 mM. Control incubations for this treatment had an equal amount of 2-propanol without antimycin A. The oxidative phosphorylation uncoupler 2,4-dinitrophenol (DNP) was used at a concentration from 0 to 1000 μM. The oxidative phosphorylation inhibitor oligomycin (1000 μg/mL stock in 2-propanol) was used at a concentration from 0 to 20 μg/mL. All solutions for this series had an equal amount of 2-propanol.

3.2.2. Calcium and calcium inhibitors (EGTA, A23187, ruthenium red)

For ruthenium red (RR) experiments, barley aleurone layers were pre-incubated in sterilized double-distilled water containing various concentrations of RR from 0 to 50 μM for 1 h followed with further treatment under either anoxia (N_2 atmosphere) or normoxia (normal air atmosphere) for 6 h.

For EGTA and A23187 experiments, freshly prepared aleurone layers were pre-incubated in sterilized double-distilled water containing various components (EGTA, 5 mM, and/or A23187, 2 μM) for 2 h (see the legends of Figure 23 and Figure 24). The solution was removed and replaced with sterilized double distilled water followed with further treatment under N_2 or air for 6 h.

3.2.3. CaM antagonist, protein phosphatase and kinase inhibitors (W-7, Okadaic acid and A3)

Barley aleurone layers were treated for 1 h followed with further treatment under N_2 (anoxia) or air (normoxia) for 6 h in the same solution. For the protein phosphatase inhibitor okadaic acid (OA) experiment, the concentrations were from 0 to 200 nM (diluted from stock 12.4 μM in ethanol; each solution had an equal amount of ethanol). A3 was used at the concentration of 0 to 200 μM (diluted from a stock of 31.2 mM in dimethyl sulfoxide (DMSO); each solution had an equal amount of DMSO). Calmodulin antagonist W-7 was used at the concentration from 0 to 1000 μM (diluted from the stock of 26.5 mM in water).

3.2.4. Hb inhibitors (Desferrioxamine, Ni^{2+}, Co^{2+} and 4,6-Dioxoheptanoic acid)

Freshly prepared barley aleurone layers were incubated in water containing various additions for 1 h followed with further incubation under N_2 or air for another 6 h. Desferrioxamine was used at the concentration of 130 μM, 4,6-dioxoheptanoic acid at 2 mM, $NiCl_2$ at 300 μM, $CoCl_2$ at 100 μM.

3.2.5. Nitrate

Barley aleurone layers were incubated with various concentration of nitrate (see Figure 21) containing 10 μg/mL of chlorophenical for 1 h followed with further treatment under N_2 or air for 6 h or stated time period. The concentration used in the experiments ranged from 0 to 100 mM.

3.3. Anaerobic treatments

3.3.1. Barley aleurone layers

Isolated barley aleurone layers were incubated under controlled oxygen tensions basically as described previously (Taylor et al., 1994). For anoxia treatment, each flask was flushed with N_2 for 1.5 min, the flasks were placed in a 3-liter jar, which was in turn purged with N_2 for 1 h. For treatments longer than 6 h, the jar was reflushed with N_2 for 30 min every 6 h thereafter. In treatments with different O_2 tension or various O_2 and CO combinations, aleurone-containing flasks were placed in 1-liter jars, which were sealed and purged with various O_2/N_2 or $CO/O_2/N_2$ mixtures to vary the O_2 or CO/O_2 partial pressure for 20 min. The gas mixtures were prepared using a gas mixing system (MKS-232, MKS Instruments, MA). The jars were re-purged with the same gas mixture every 4 h thereafter. Samples were harvested at appropriate time points.

3.3.2. Barley coleoptiles

Young seedlings (2-3 days after being placed on the germinating box) with the coleoptiles approximately 2 cm in length were set in a 5-liter desiccator, which was then flushed with pure N_2 (99.9 %) for 1 h and then sealed. The jar was repurged with the nitrogen gas for 30 min every 6 h thereafter. Samples were collected at appropriate time points.

3.3.3. Barley and maize seedlings (flooding; anoxia)

Three-leaf-stage seedlings of barley and maize grown in pots were placed in a container with a sufficient amount of water to cover the pot surface. Barley and maize roots were collected at different time points of waterlogging (flooding).

In one experiment (Figure 10), 3-leaf-stage barley seedlings were set in a 5-liter jar, which was then purged with N_2 for 1 hour. The jar was re-flushed with N_2 for 30 min every 6 h thereafter.

3.4. Southern blotting

3.4.1. DNA isolation

Plant material was frozen and ground with a mortar and pestle in liquid nitrogen. For every 5 g weight of fresh material, 10 mL of 60°C pre-warmed cetyltrimethylammonium bromide (CTAB) buffer (see Appendix I) was added (for monocots 15 mL of CTAB buffer was added for every 5 g fresh material) and incubated in 60°C water bath for 1 h. The tube was gently inverted every 15 min. Ten milliliters of chloroform (24 chloroform : 1 isoamyl alcohol, v/v) was added and centrifuged at room temperature in SS-34 rotor at 700 rpm for 10 minutes. Chloroform extraction was repeated once more. To the aqueous phase, 0.1 volumes of 5 M ammonium acetate and 2 volumes of isopropanol were added. After 15 min at room temperature, the DNA was recovered by centrifugation. Proteins are not precipitated by isopropanol in the presence of 2 M ammonium acetate. The pellet was washed with 70% ethanol 2-3 times and resuspended in 5.0 mL of TE buffer (see Appendix I). Five hundred micrograms of RNase A (boiled for 10 min previously to eliminate any trace DNase) was added, and the resulting solution was incubated at 37°C for 60 min. After adding an equal volume of phenol (Tris-HCl saturated, pH 7.5), the solution was vortexed and centrifuged at 4000 rpm (SS-34 rotor) for 5 min. The aqueous phase was extracted once with phenol:chloroform (1:1) and once more with chloroform (24 chloroform : 1 isoamyl alcohol, v/v) and centrifuged at 4000 rpm (SS-34 rotor) for 5 minutes. DNA was

precipitated as described above. The resulting DNA pellet was dissolved in a small amount of TE buffer (see Appendix I).

3.4.2. Probe preparation

Probes (cDNA inserts isolated from the vectors) were labeled with [α-^{32}P]dCTP according to Sambrook *et al* (1989). Hb probe was barley Hb cDNA (Taylor et al., 1994), barley *Adh* 1 probe was a gift from A. Good, barley *Ldh* probe was a gift from A. D. Hanson. Ribosomal DNA probe was used to indicate equal loadings.

3.4.3. DNA hybridization

Ten micrograms of DNA was digested overnight with 1 µL desired restriction endonuclease (*EcoRI*, *HindIII*, *BamHI* and *KpnI*) and proper buffer (One-Phor-All, Pharmacia). After electrophoresis in a 1% (w/v) agarose gel with TAE buffer (see Appendix I), the DNA was denatured in 0.2 M HCl and followed with incubation in the denaturing solution (1.5 M NaCl, 0.5 M NaOH), and it was finally neutralized in the neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.0). The gel was blotted to Hybond-N$^+$ (Amersham) with 0.4 M NaOH for 12 h at room temperature.

The membrane was prehybridized in the hybridization buffer (6 x SSC, 5 x Denhardt's solution, 1% SDS, w/v, and 100 µg/mL salmon sperm DNA) (see Appendix I for SSC and Denhardt's) at 65°C for 2 h. Then, ^{32}P-labeled probe was added to the hybridization solution and incubated at the same temperature for 14 to 16 h. The membranes were then washed at 65°C for 30 min in 2 x SSC plus 0.5% (w/v) SDS, then 20 min in 2 x SSC plus 0.1% (w/v) SDS and 5 to

10 min in 0.2 x SSC plus 0.1% (w/v) SDS at 65°C. The membranes were wrapped and placed in a cassette with enhancing screens (DuPont) and exposed to Kodak XAR-5 film at -75°C for an appropriate time period.

3.5. Northern blotting

3.5.1. RNA isolation

Total RNA for northern analysis was isolated from 50 aleurone layers or approximately 3 to 4 g of fresh weight tissue. The tissue was ground in liquid nitrogen, and incubated with 60°C preheated 10 mL extraction buffer (0.2 M Na-acetate. 10 mM EDTA, 0.2% SDS, 0.1 M β-mercaptoethanol, pH 5.2) and 10 mL water-saturated phenol. The resulting solution was homogenized at speed 5 with a Polytron Homogenizer (Brinkmann) for 1 min followed with centrifugation at 6000 rpm (SS-34 rotor) for 15 min. The aqueous phase was collected and extracted with one volume of phenol/chloroform (1/1, v/v). After being vortexed and centrifuged at 6000 rpm (SS-34 rotor), the aqueous phase was collected and extracted with one volume of chloroform (chloroform/isoamyl alcohol, 24:1, v/v). LiCl was added to the collected aqueous phase to make a final concentration of 2 M. After being placed at 4°C overnight, it was centrifuged at 10,000 rpm (SS-34 rotor) at 4°C for 30 min. The pellet was air dried and then dissolved in 400 μL of sterilized-double-distilled water. The RNA was further purified by one phenol/chloroform and two to four chloroform (24 chloroform : 1 isoamyl alcohol, v/v) extractions. The resulting aqueous phase was collected, and Na-Acetate was added to a final concentration of 0.3 M. After being set at -20°C for at least 4 h, it was centrifuged at 13,000 rpm

in the mini-centrifuge at 4°C. The resulting pellet was air dried and dissolved in 20 to 40 µL sterilized double-distilled H_2O. Total RNA was quantified according to its absorbance at 260nm.

3.5.2. RNA hybridization

Unless otherwise indicated, 10 µg total RNA were denatured at 65°C for 18 min in a final volume of 20 µL containing 4 µL formaldehyde and 10 µL formamide and then chilled on ice (Sambrook et al., 1989), electrophoresed with RNA running buffer (see Appendix I) on a 1.25% agarose gel containing 2.2 M formaldehyde and a trace of ethidium bromide, and finally transferred to Hybond N^+ membranes (Amersham) with 20 x SSC (see Appendix I) or 0.05 M NaOH for 10 - 15 h. Membranes that were blotted with 20 x SSC were air dried for 1 h before UV cross-linked on a transilluminator for 3 min. For membranes blotted with NaOH, no UV cross-linking was needed. Prehybridization and hybridization were carried out for 2 and 14 to 16 h, respectively, at 65°C in hybridization buffer containing 6 x SSC, 5 x Denhardt's solution, 1% (w/v) SDS, and 100 µg/mL salmon sperm DNA. The membranes were washed at 65°C for 30 min in 2 x SSC, 0.5% (w/v) SDS, then 20 min in 2 x SSC, 0.1% (w/v) SDS and 5 to 10 min in 0.2 x SSC, 0.1% (w/v) SDS at 65°C. The membranes were exposed to Kodak XAR-5 film at -75°C as described in Southern Blotting (3.4.3). After being stripped by boiling the filter in 0.1% SDS (w/v) for 5 min, the membrane was reprobed with another probe and autoradiographied. The resulting signals (northern blotting bands in the X-film) were quantified by scanning the intensity and area of the band with an imaging system (Imagex).

3.6. Western blotting

3.6.1. Barley hemoglobin antibody

Barley Hb antibody was developed in rabbits (Duff & Hill, unpublished data). The anti-serum was collected from the rabbits, and used as Hb antibody against hemoglobin in protein-immuno analysis.

3.6.2. Protein isolation and separation

Fifty barley aleurone layers or 2 to 4 g of other plant tissue was ground in 3 mL of ice-cold protein extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and freshly prepared 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]) with pre-cooled motors and pestles. The extracts were centrifuged at 10,000 rpm (SS-34 rotor) at 4°C for 20 min. The supernatants were transferred into a 1.5 mL microcentrafuge tube followed by centrifuged at 13,000 rpm at 4°C for 30 min. The supernatants were collected and the protein was quantified by the Bradford protein assay (Bradford, 1976).

3.6.3. Blotting

Thirty micrograms of total protein were denatured by adding 0.25 volumes of loading buffer (0.05 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 3% β-mercaptoethanol and a trace of bromophenol blue) and incubated at 100°C for 5 min. After separating proteins by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) (15%) (for reference, see Duff et al., 1997), the gel was then soaked together with nitrocellulose membrane in transferring buffer (25mM Tris, 190 mM glycine, 20% methanol). Proteins in the gel were transferred to the

membrane. The membrane was then incubated in 3% (w/v) powdered milk or gelatin in TBS buffer (see Appendix I) at room temperature (powdered milk) or 37°C (gelatin) for at least 1 h. The membrane was then washed twice with TBS buffer , and then incubated with crude immune serum (Hb antibody) (1:3000) in 1% powdered milk in TTBS buffer (see Appendix I) for at least 2 h. After washing three times with TTBS buffer , the membrane was incubated with the second antibody (anti-rabbit IgG conjugated with alkaline phosphatase, 1:3000) in 1% powdered milk in TTBS buffer at room temperature for at least 1 h. Thereafter, the membrane was developed by adding nitroblue tetrazolium (NBT) and bromo-4-chloro-3-indolyl phosphate (BCIP) to final concentrations of 3.3 mg/mL and 1.65 mg/mL, respectively.

3.7. Oxygen uptake measurement

Oxygen uptake was measured polarographically with a Clark oxygen electrode for 5 min. The typical incubation medium contained 1.5 mL of 0.1 M phosphate buffer (pH 7.0), 7 barley aleurone layers and various additions.

3.8. Enzyme activity assay: ADH and LDH

Ten barley aleurone layers were ground with sand in an ice-cooled mortar and pestle in 2.0 mL of 0.15 M Tris-HCl (pH 8.0) containing 10 mM dithiothreitol (DTT). The extract was centrifuged at 10000 rpm (SS-34 rotor) for 30 min at 4°C, and the supernatant was taken for spectrophotometric assays of LDH and ADH.

LDH was assayed using the approach of Hanson & Jacobsen (1984), in the pyruvate--lactate direction, monitoring pyruvate-dependent NADH oxidation at 340 nm at pH 8.0 in the

presence of a pyrazole ADH inhibitor (Hanson & Jacobsen, 1984). At a concentration of 10 mM in the reaction mix, 4-bromopyrazole suppressed ADH activity completely. The assay mix (final volume 3.0 mL) contained 1 mL of 0.5 M Tris-HCl (pH 8.0), 400 µg NADH, 30 µmole 4-bromopyrazole, 30 µmole sodium pyruvate, and 0.4 mL enzyme extract. The assay was carried out at 27°C.

ADH activity was assayed in the ethanol to acetaldehyde direction. The reaction mix (final volume 3.0 mL) contained 1 mL of 0.5 M Tris-HCl (pH 9.0), 100 µmole ethanol, 3 µmol NAD, 0.1 mL enzyme extract. The assay was carried out at 27°C. ADH and LDH activities are reported in International Units (IU, µmol/min.mg protein).

3.9. Mitochondrial isolation

Ten grams of etiolated coleoptiles were ground in 25 mL of ice-cold grind medium (0.5 M mannitol, 10 mM K_2HPO_4, 2 mM cysteine, 1 mM EDTA, 0.1 % BSA, w/v, pH 7.5) in an ice-cold motar and pestle. The brei was removed by being filtered through Nitex nylon cloth (50 µm openings). The remaining extract was centrifuged at 1000*g* for 5 min. The supernatant was collected and centrifuged at 20,000*g* for 5 min. The pellet was resuspended in approximately 2 mL grind medium and layered on top of a Percoll gradient, and then centrifuged at 7500*g* for 30 min. The top layers down to the mitochondria were removed and the bottom layer was removed, leaving the purified mitochondria band. Ten millilitres of grind medium was added and the mitochondria were spun down at 20,000*g* for 5 min. The supernatant was removed and the

purified mitochondria could be re-suspended in grind medium.

Percoll gradient

Percoll solutions: (1) 1.35 mL Percoll diluted to 10 mL with gradient buffer

(2) 2.10 mL Percoll diluted to 10 mL with gradient buffer

(3) 2.25 mL Percoll diluted to 5 mL with gradient buffer

Gradient buffer: 0.25 M sucrose, 0.2 %(w/v) BSA, 10 mM K_2HPO_4 (pH 7.5)

Gradient preparation: place 2 mL of (3) to the bottom of a centrifuge tube, carefully layer 4 mL of (2) on top of (3) ; and then layer 4 mL of (1) on top of (2).

4. Results

The major results regarding the regulation and possible function of Hb in barley, particularly in barley aleurone layers, will be presented in the order of (1) Hb occurrence in plants, (2) Hb gene expression is different barley tissues, (3) Hb gene expression in response to oxygen shortage, (4) Hb gene expression in relation to oxidation respiration, and (5) Hb gene expression and signal transduction in barley aleurone tissue.

4.1. Hemoglobin gene distribution in several plant species

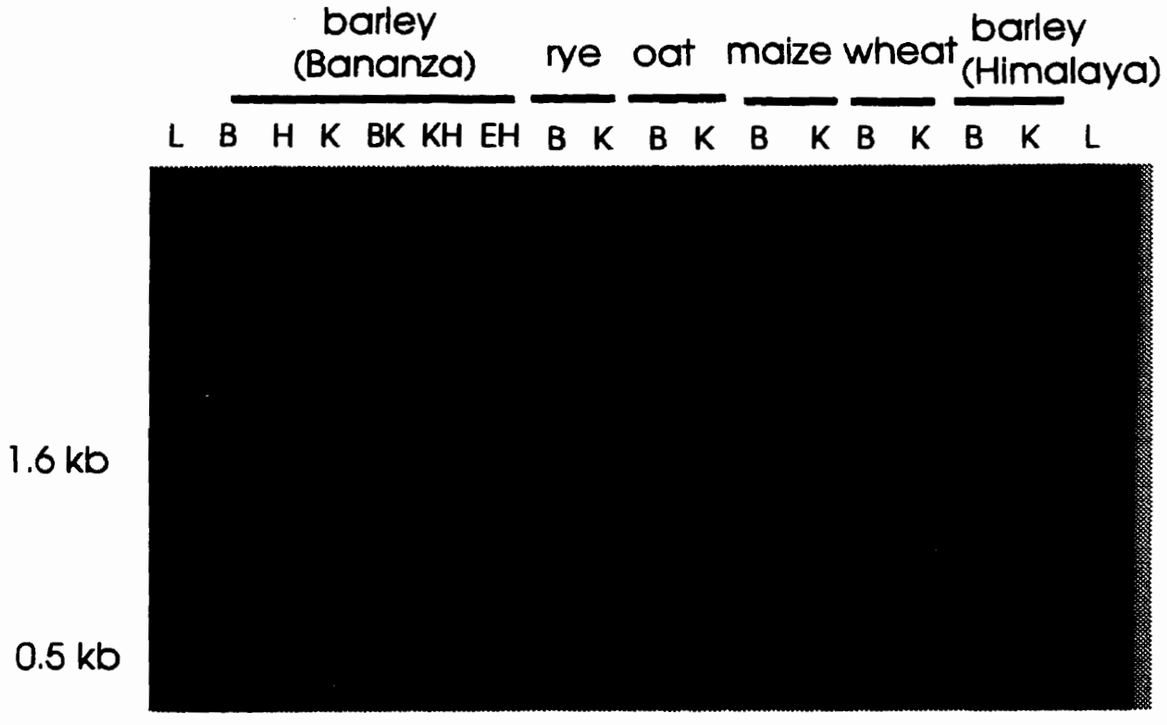
A previous study indicated that the Hb genes exist in barley, maize, rye and wheat (Taylor et al., 1994). A full length Hb cDNA (approximately 1 kb) probe of barley was used to detect the presence of Hb in other plant species. Southern blot analysis showed that one to three hybridization bands appeared in the genomic DNA isolated from barley, rye, oat, maize and wheat (Fig. 3a). Several hybridization bands appeared from rice genomic DNA digested with different restriction endonucleases (Fig. 3b), indicating that closely related Hb sequences exist in rice, too. One hybridization band was observed from bean genomic DNA. One faint band was also present in the DNA isolated from *Brassica napus*.

4.2. Hb gene expression is tissue specific under normal growth conditions

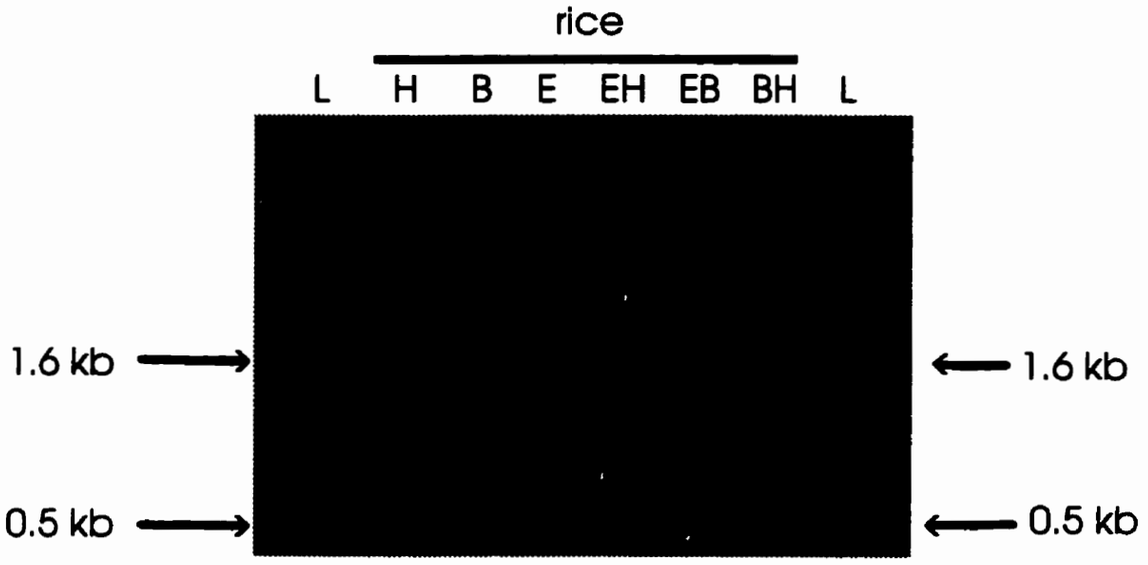
Northern analysis of RNA isolated from different tissues in barley indicated that Hb transcription is tissue specific. Approximately a 1- kb hybridization band was present in the RNA isolated from roots, coleoptiles and aleurone layers, but was absent in the RNA isolated from leaves (Fig . 4).

Figure 3. Southern blot analysis of various plants. Ten micrograms of genomic DNA isolated from leaves were digested with restriction endonuclease, electrophoresed in 1% agarose gel, blotted to Hybond-N^+ (Amersham), and probed with a full-length ^{32}P-labelled barley Hb cDNA as described in "Material and Methods". B, H, K and E represent *BamHI*, *HindIII*, *KpnI* and *EcoRI*, respectively. L represents the 1-kb ladder DNA (Canadian Life Technologies). BK: *BamHI* + *KpnI*, EH: *EcoRI* + *HindIII*, KH: *KpnI* + *HindIII*, EB: *EcoRI* + *BamHI*, BH:*BamHI* + *HindIII*. a, barley, rye, oat, maize and wheat. b, rice (top), and bean and rapeseed (bottom).

a


barley (Bananza)
rye
oat
maize
wheat
barley (Himalaya)
L B H K BK KH EH B K B K B K B K B K L
1.6 kb
0.5 kb

b


rice
L H B E EH EB BH L
1.6 kb
0.5 kb
1.6 kb
0.5 kb

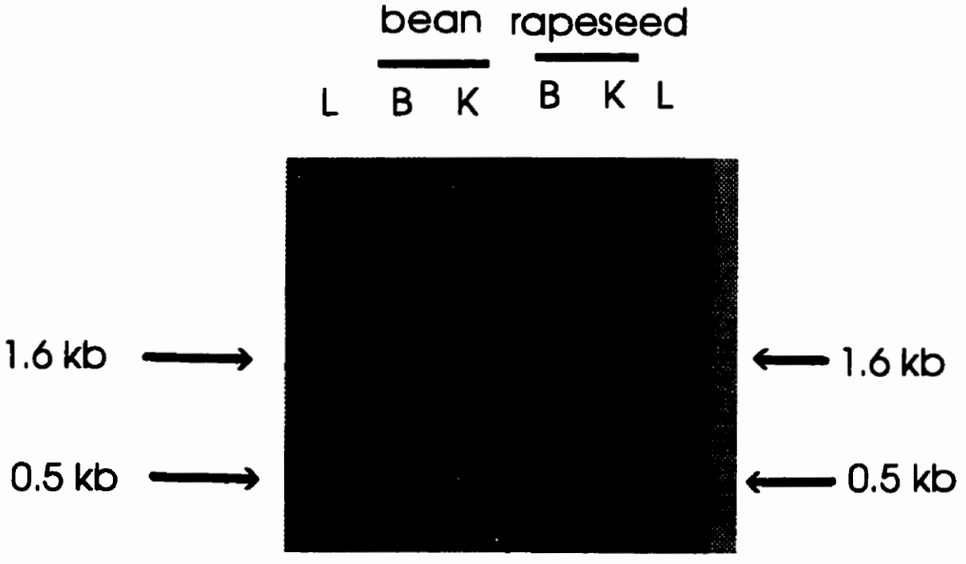
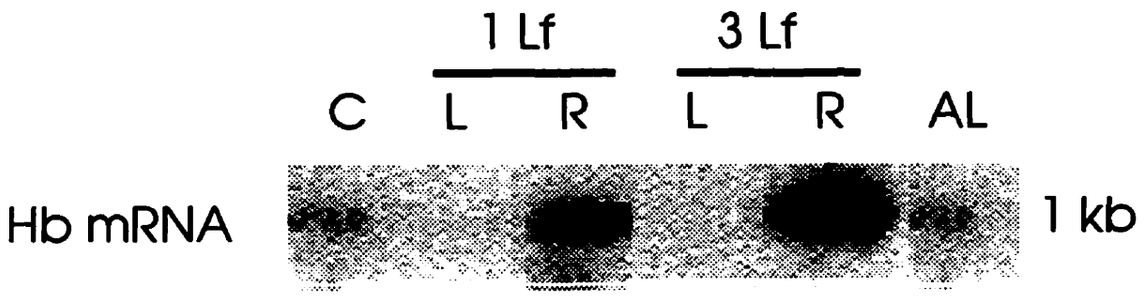

bean rapeseed
L B K B K L
1.6 kb
0.5 kb
1.6 kb
0.5 kb

Figure 4. Hb gene expression in different barley tissues. Ten micrograms of total RNA was electrophoresed in 1.25% denaturing agarose/formaldehyde gel, blotted to a Hybond-N^+ filter, and probed with the full-length barley Hb cDNA probe as described in "Materials and Methods". C, L, R and AL represent coleoptiles, leaves, roots and aleurone layers, respectively. 1 Lf : 1-leaf stage; and 3 Lf :3-leaf stage.


1 Lf
3 Lf
C
L
R
L
R
AL
Hb mRNA
1 kb

4.3. Hb gene is induced by oxygen shortage

4.3.1. Anaerobic stress and Hb gene expression in barley aleurone layers

Previous studies have shown that Hb is induced in barley aleurone layers by oxygen deprivation (Taylor et al., 1994). A relatively faint band was observed from RNA isolated from aleurone layers which were incubated under higher oxygen tension (>10%) (Fig. 5). However, as the oxygen tension was decreased to 5%, Hb mRNA level was enhanced. Further induction was observed as the oxygen concentration was lowered further to 2%, and finally to 0%. The results indicated that Hb transcription is activated by oxygen shortage.

To investigate the induction kinetics of Hb under anaerobic conditions, barley aleurone layers were placed under anoxia (N_2) for various time periods. As shown in Fig. 6a, Hb mRNA level started to increase within 1 h of anaerobiosis. It was then greatly increased as anoxia treatment continued to 12 h, when the transcripts reached their maximum level in anoxia. This level, which is about 50 times of that at 0 h, was maintained at prolonged anaerobiosis until 72 h (Fig. 6b), the longest period of anoxia tested in this investigation. The results indicate that Hb transcription is an early and also a pronounced event in barley aleurone tissue subjected to oxygen deprivation.

Western blot analysis of Hb from anoxia-treated barley aleurone layers indicates that Hb induction also occurred on the protein level (Fig. 7a). From the blot, about twice the Hb was observed in anoxia-treated (lane 2 in 'Figure 7a') over that of untreated aleurone layers (lane 3 in 'Figure 7a'), indicating that not only Hb transcripts but also Hb protein were induced by oxygen deficit. However, the induction of Hb protein appeared not be as pronounced as that in transcripts shown above.

Figure 5. Hb, Adh and Ldh gene expression in barley aleurone layers in response to various O_2 tensions. Ten micrograms of total RNA were electrophoresed in denaturing 1.25% agarose gel containing a trace (5 mg/L) of ethidium bromide. Loading equivalence and RNA integrity were confirmed prior to transfer by UV visualization of ethidium bromide-RNA complex. RNA was blotted to a Hybond-N^+ membrane. The blot was probed with ^{32}P-labelled barley Hb cDNA, the barley *Adh*1 probe and the *Ldh*1 probe consecutively with the former probe removed by stripping the membrane as described in "Materials and Methods". The numbers shown below the blots are the intensities and areas of the corresponding bands, obtained by scanning the blots using an imaging system (Imagex). ND-not detectable.

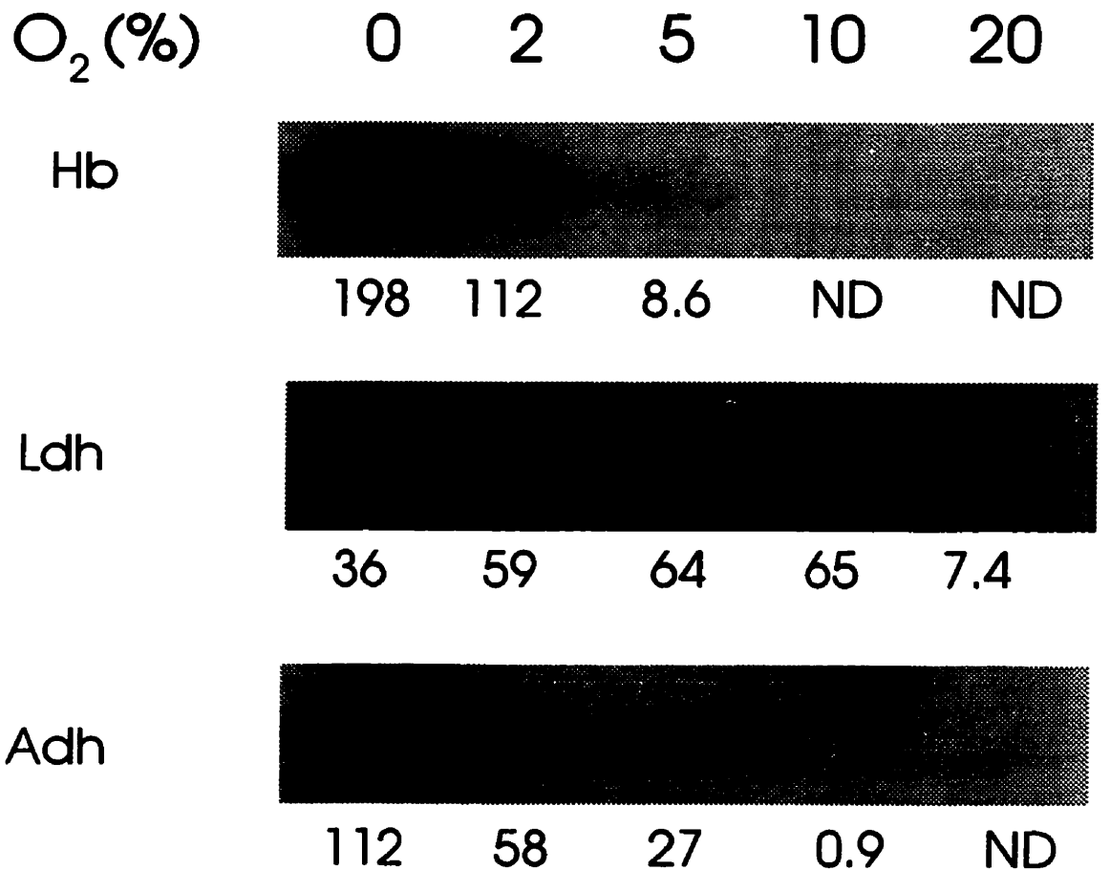
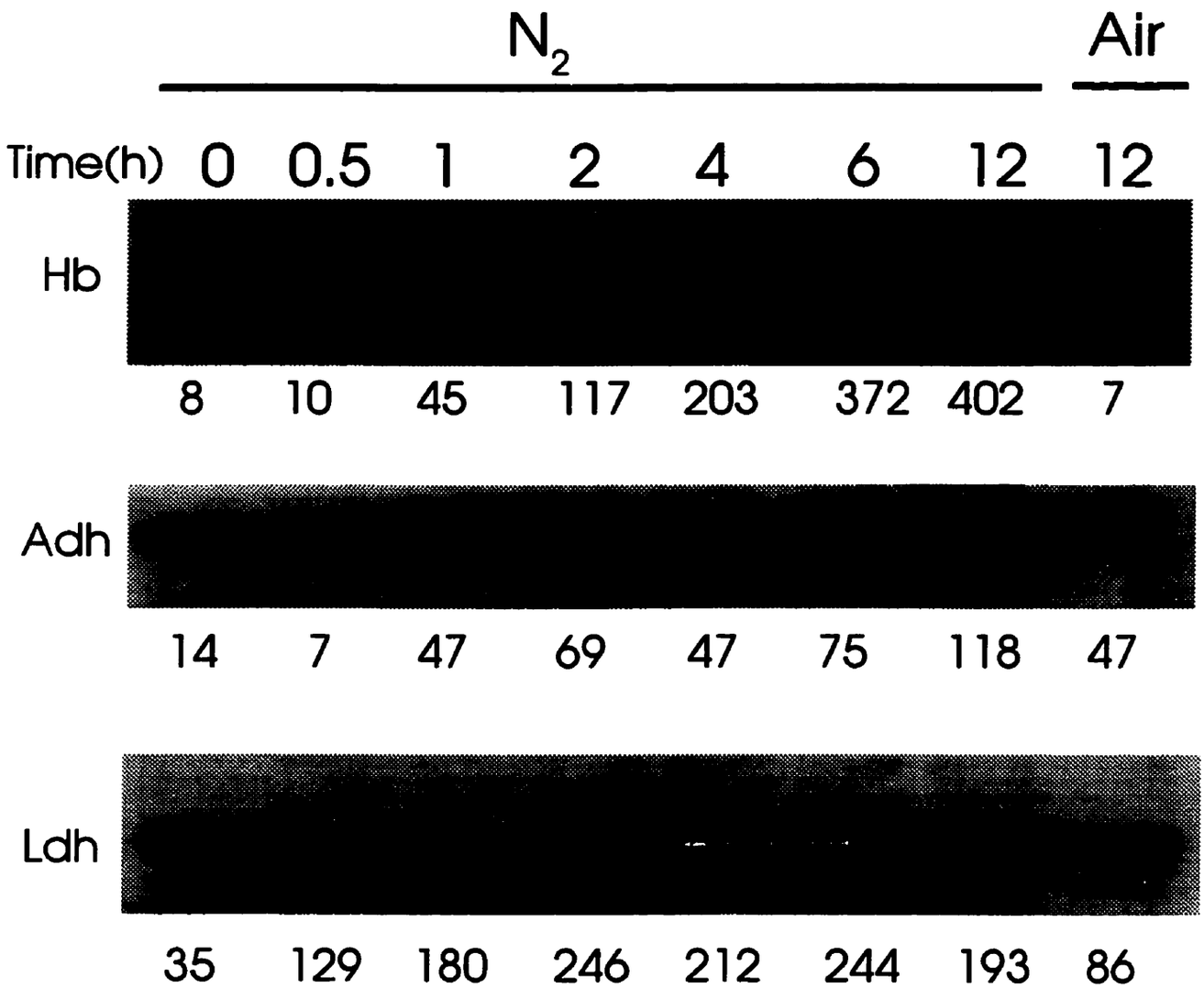
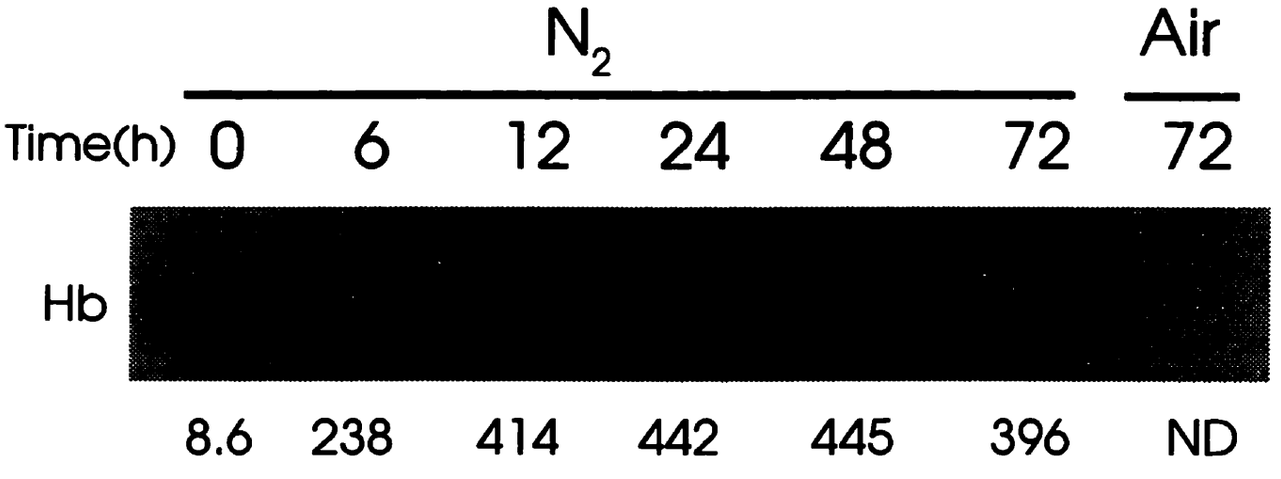

O_2 (%)
0
2
5
10
20
Hb
198
112
8.6
ND
ND
Ldh
36
59
64
65
7.4
Adh
112
58
27
0.9
ND

Figure 6. Induction of Hb, Adh and Ldh in barley aleurone layers follow various periods of anoxia. Barley aleurone layers were treated under anoxia (N_2) for different periods of time. Total RNA was isolated. Ten micrograms of total RNA were electrophoresed in denaturing 1.25% agarose gel containing a trace (5 mg/L) of ethidium bromide. Northern blot analysis was carried out with Hb, *Adh*1 and *Ldh*1 probes as described in Figure 5. *a*, 0 - 12 h. *b*, 0-72 h. The numbers shown below the blots were the intensities and areas of the corresponding bands, obtained by scanning the signals using an image analysis system (Imagex). ND-not detectable.

a


N2
Air
Time(h) 0 0.5 1 2 4 6 12 12
Hb
8 10 45 117 203 372 402 7
Adh
14 7 47 69 47 75 118 47
Ldh
35 129 180 246 212 244 193 86

b



Studies on the time-course of Hb protein induction in anoxia indicate that Hb protein started to increase within 6 h of anaerobiosis (Fig. 7b), which was about 4 to 5 h after the mRNA levels began to increase. Hb protein continuously increased until approximately 24 h of anaerobiosis, when it reached its maximum level. This level was maintained during the rest of period under anoxia. Even though Hb induction can be noticed in both messenger RNA and protein level, the transcripts appeared to be more greatly affected by oxygen deprivation than was the protein.

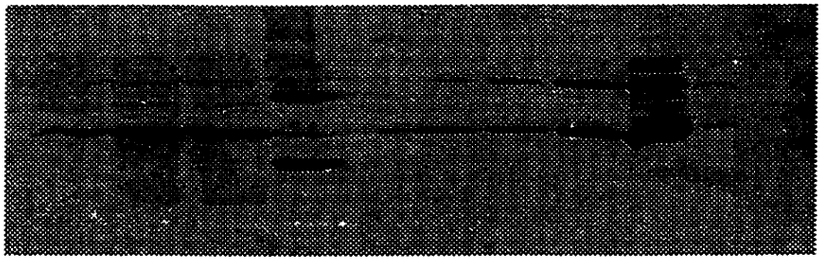
After 24 h of anaerobiosis, when both mRNA and protein reached their highest levels (Fig. 6 and Fig. 7), barley aleurone layers were placed under normal air, in which oxygen concentration was about 20%. Northern blot analysis (Fig. 8) indicates that Hb transcript levels started to decrease after 4 to 6 h of returning to aerated conditions. The transcripts continuously decreased until they reached the background level of the un-induced aleurone layers after about 24 h. Western blot analysis shows that Hb protein level began to decrease 2 to 4 h after the aleurone layers were returned to normal air atmosphere (Fig. 8). However, unlike mRNA, Hb protein was maintained to a certain level which was apparently higher than that in un-induced aleurone tissue, indicating that the half-life of Hb protein is longer than its mRNA counterpart.

4.3.2. Anaerobic stress and Hb gene expression in other tissues

Coleoptiles were used to investigate the kinetics of both Hb mRNA and protein induction. From Fig. 9, it can be noted that Hb mRNA bands were too faint to be detected in aerated coleoptiles, whereas the bands were easily observed in the absence of O_2 (under N_2). Six hours of anaerobiosis strongly induced Hb mRNA. Further incubation under anoxia stimulated Hb gene

Figure 7. Western blot analysis of Hb in barley aleurone layers subjected to anoxia. Total proteins were isolated from barley aleurone layers (all lanes in *b*, lane 2 and 3 in *a*) and maize root (lane 1 in *a*). Thirty micrograms of protein were separated on SDS-PAGE. The protein was transferred to nitrocellulose filter, and probed with the barley Hb antibody developed in rabbits. *a*, 1-maize roots subjected to waterlogging, 2-barley aleurone layers subjected to 24 h of N_2, 3-barley aleurone layers in air (24h), 4-standard proteins (Rainbow markers, Amersham), 5 to 9 represent a series of concentrations (0.025, 0.05, 0.125, 0.25 and 2.5 µg, respectively) of recombinant barley Hb. *b*, time course (h) of Hb protein in response to anoxia (N_2) in barley aleurone layers. The numbers shown below the blots are intensities and areas of the corresponding bands analysed by an image analysis system (Imagex).

a


1 2 3 4 5 6 7 8 9
5.4 24 13 \ 0.4 8 16 44 120

b

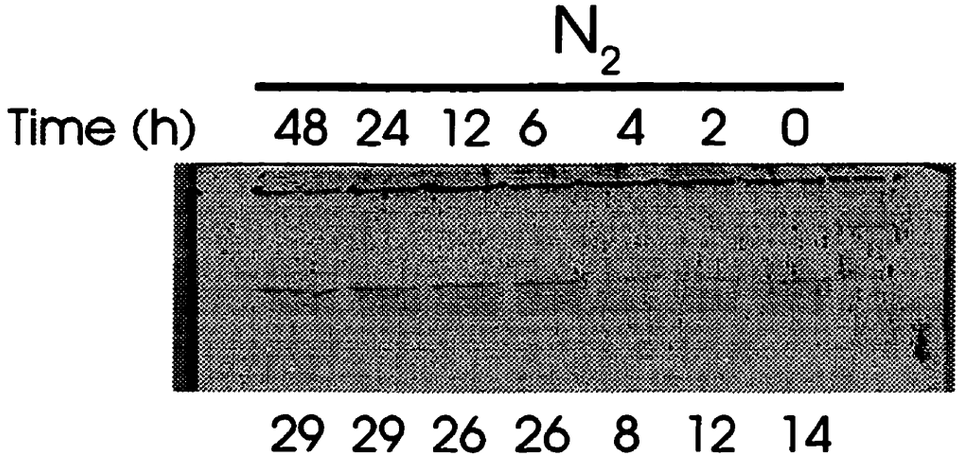

N2
Time (h) 48 24 12 6 4 2 0
29 29 26 26 8 12 14

Figure 8. Reversal of induction of Hb in barley aleurone layers. Barley aleurone layers were treated under anoxia (N_2) for 24 h first, and then set back to aerobic conditions (air) for various periods of time (0-24 h). Total RNA and protein were isolated separately. Northern blot and Western blot analysis were carried out and presented as described in "Materials and Methods" and previous figures.

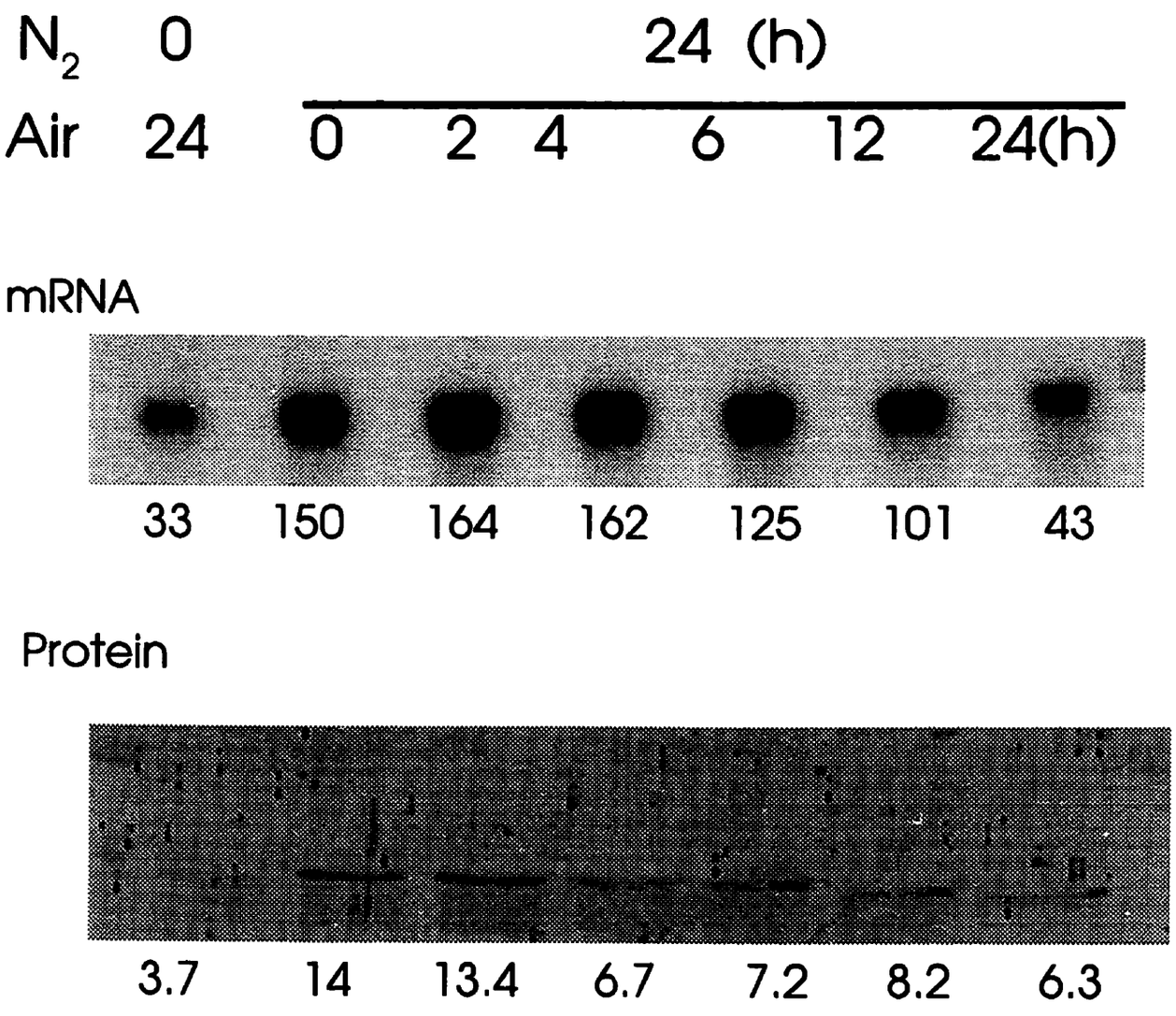
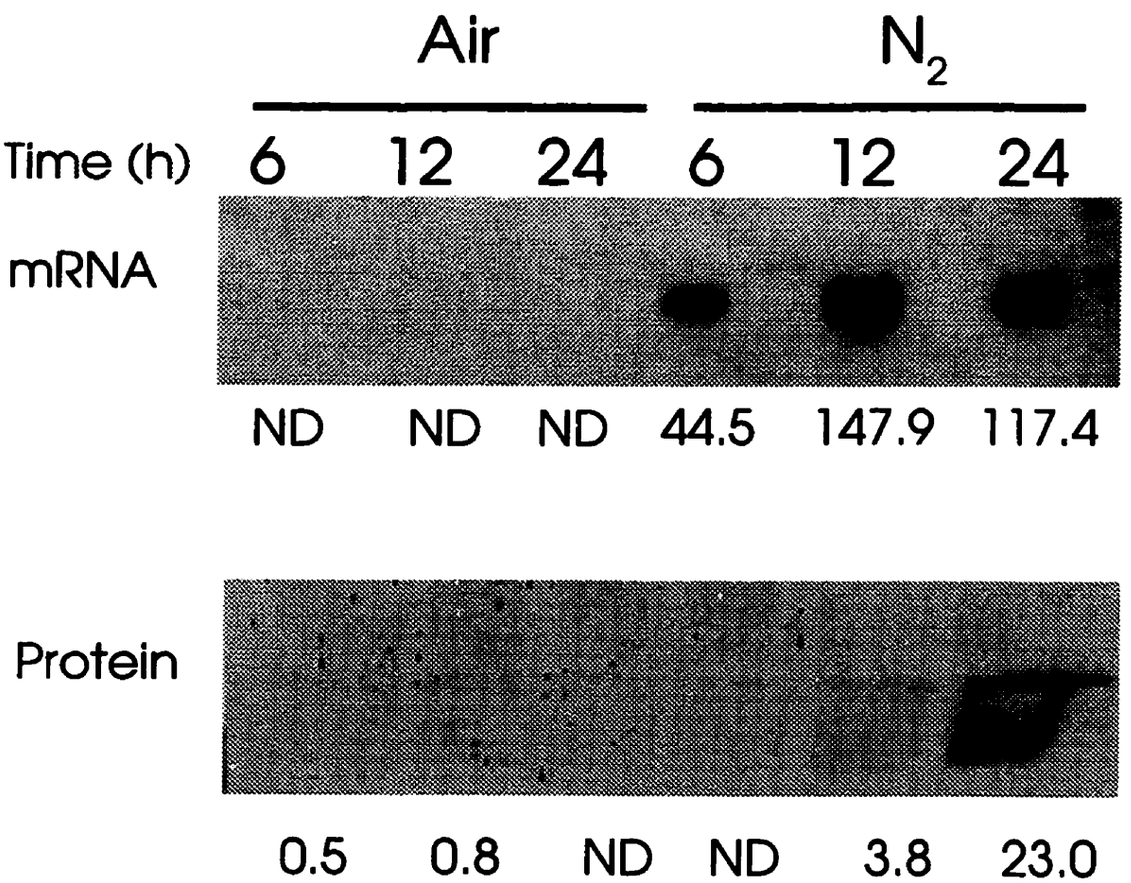

N_2 0
24 (h)
Air 24
0 2 4 6 12 24(h)
mRNA
33 150 164 162 125 101 43
Protein
3.7 14 13.4 6.7 7.2 8.2 6.3

Figure 9. Induction of Hb in coleoptiles under anoxia. Young seedlings with approximately 2-cm-long coleoptiles were set under air or N_2 for 6, 12 and 24 h. RNA and protein were extracted separately from the treated coleoptiles. Northern and Western blot analysis were performed and presented as described in "Materials and Methods" and previous figures. The number below the blot are intensities and areas of the corresponding bands, obtained by scanning the blot using an image analysis system (Imagex). ND-not detectable.


Air
N_2
Time (h)
6
12
24
6
12
24
mRNA
ND
ND
ND
44.5
147.9
117.4
Protein
0.5
0.8
ND
ND
3.8
23.0

expression: 12 h of anoxia resulted in 2-3 fold of mRNA over that of 6 h anoxia. However, 24 h anaerobiosis did not appear to affect mRNA levels further: not very much difference was noticed in both 12 h and 24 h time periods under anoxia.

Western blot analysis of Hb in coleoptiles shows that Hb protein level did not increase until 12 h of anaerobiosis (Fig. 9), at least 6 h after the corresponding increase in mRNA levels. After 24 h under anoxia, Hb protein in coleoptiles had increased dramatically.

Hb gene expression in barley leaves was studied in aerated and anoxic conditions. As mentioned above, no Hb mRNA was detected in leaves from aerated plants (Fig. 4, and Fig. 10, too). When the plants were set under anoxic conditions (N_2) for a certain period of time (24 h), the plant leaves were wilted due to oxygen deprivation. Northern blot analysis of RNA isolated from the leaves and roots indicates that a Hb-like RNA band was present in the RNA isolated from the wilted leaves (24 h of anoxia) and un-treated roots (Air) (Fig. 10). However, more experiments are needed to verify whether or not this is actually a Hb band from the leaves, because hybridization specificity is decreased due to the large amount (40 µg) of RNA loaded, and also due to RNA degradation resulted from anaerobic injury of the leaves.

It has been reported that Hb mRNA is greatly induced by waterlogging in both barley and maize roots (Taylor *et al.*, 1994). In barley roots (Fig. 11), Hb transcription was dramatically induced within 12 h of flooding. It reached its maximum level at 24 h, and thereafter was maintained at the level until 72 h, the longest time tested in the study, whereas in maize roots (Fig. 12), Hb transcription was increased within 12 of waterlogging. However, as time went by, Hb mRNA levels appeared to decrease. Very similar observations were reported in an independent study in maize (Silva-Cārdenas, 1997).

Figure 10. Hb mRNA in barley leaves subjected to anoxia. Three-leaf-stage barley seedlings were treated under N_2 for 8 and 24 h, respectively. Total RNA was isolated from roots (un-treated) and leaves. Forty micrograms of total RNA were loaded and electrophoresed in 1.25% denaturing agarose gel. RNA was blotted to Hybond-N^+ membrane. The filter was hybridized with the full-length barley Hb cDNA probe. ND-not detectable.

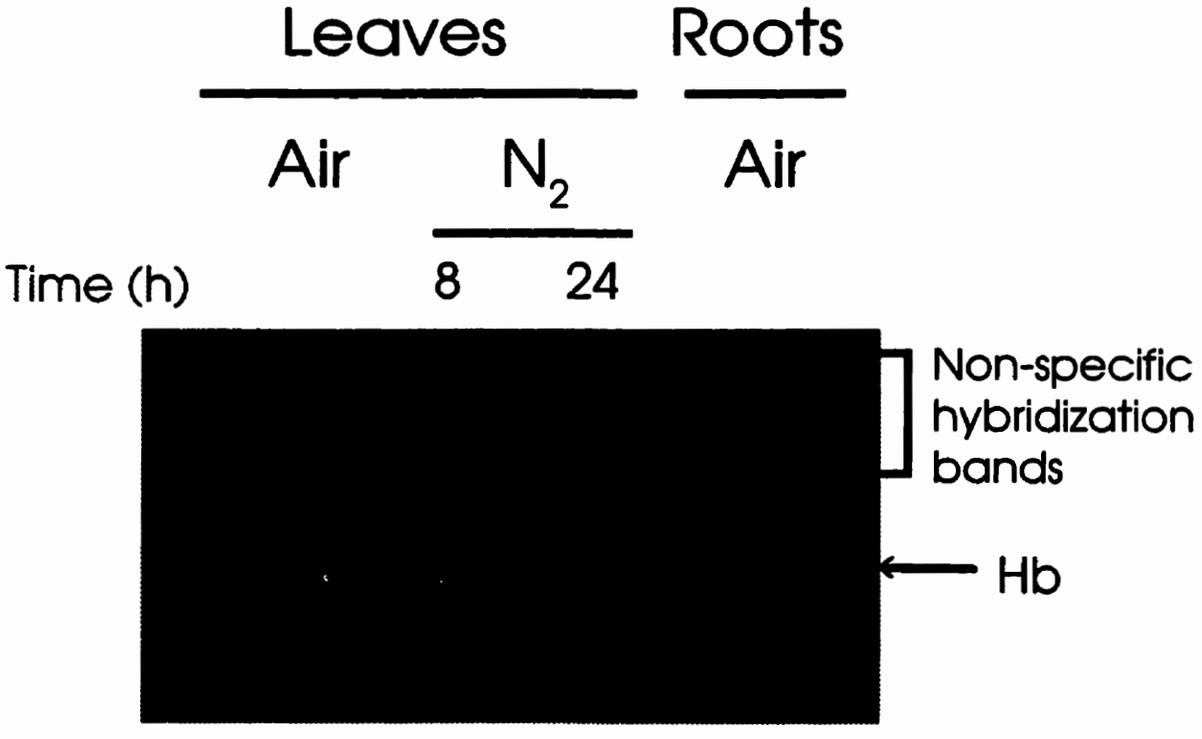

Leaves
Roots
Air
N_2
Air
Time (h)
8
24
Non-specific hybridization bands
Hb

Figure 11. Effect of waterlogging on Hb gene expression in barley roots. Three-leaf-stage barley seedlings planted in pots were submerged in tap-water for different periods of time (0 to 72 h). Roots were harvested, and total RNA was extracted. Ten micrograms of total RNA was loaded and electrophoresed in 1.25% denaturing agarose gel. After being blotted to Hybond-N^+ membrane, the RNA was probed with ^{32}P-labelled barley Hb cDNA. Loading equivalence and RNA integrity were confirmed prior to the blotting as described in "Materials and Methods". The numbers below the blot are intensities and areas of the corresponding bands, obtained by scanning the blot using an image analysis system (Imagex).

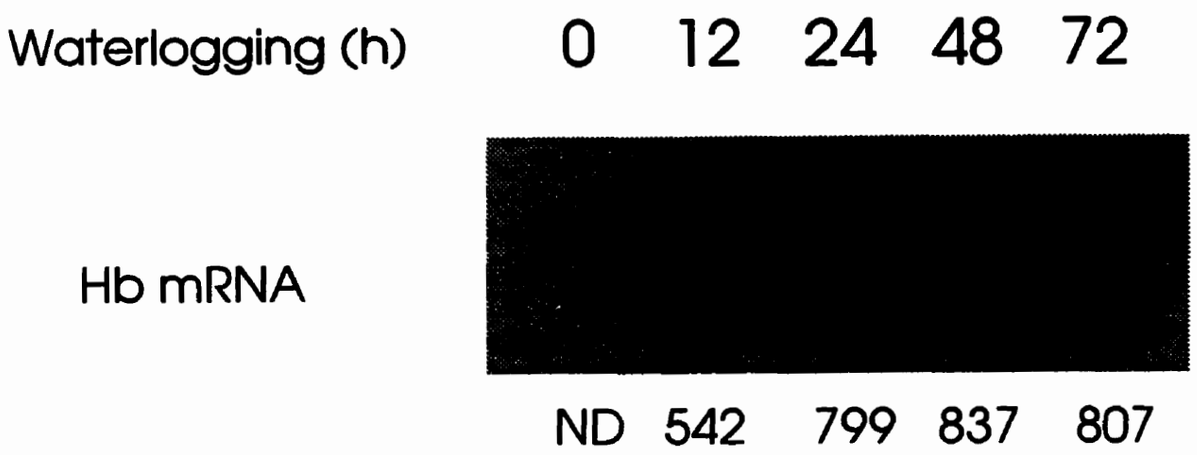
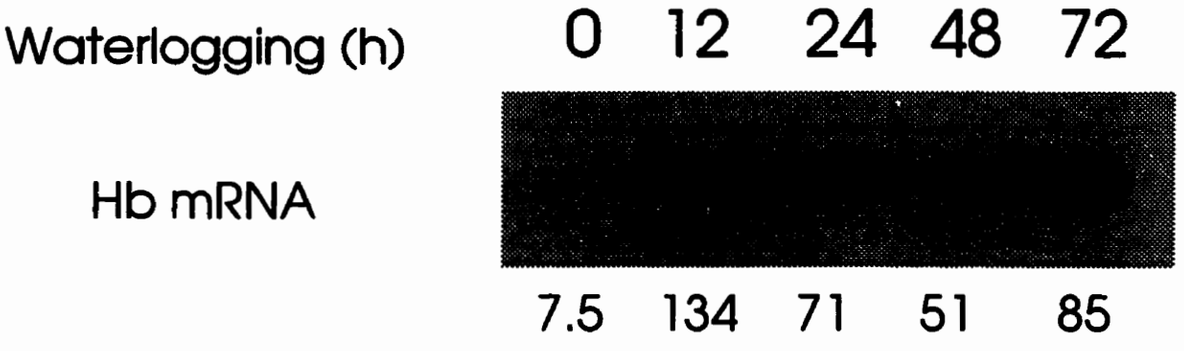

Waterlogging (h)
0 12 24 48 72
Hb mRNA
ND 542 799 837 807

Figure 12. Effect of waterlogging on Hb gene expression in maize roots. Three-leaf-stage maize seedlings grown in pots were submerged in tap-water for various periods of time (0 to 72 h). Total RNA isolated from the roots.Ten micrograms of total RNA was loaded and electrophoresed in 1.25% denaturing agarose gel. Northern blot analysis with a ^{32}P-labelled barley Hb cDNA was carried out and presented as described in "Materials and Methods" and in Figure 11.


Waterlogging (h)
0 12 24 48 72
Hb mRNA
7.5 134 71 51 85

4.3.3. *Adh* and *Ldh* gene expression and enzyme activities

Alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) transcripts were induced by oxygen deprivation in barley aleurone tissue (Fig. 5 and Fig. 6a). Studies on the induction kinetics of both *Adh* and *Ldh* mRNA indicate that they were induced within 1 h of anaerobiosis. The *Adh* transcripts reached the highest level, which is about 8-fold of that at 0 h, around 12 h of anaerobiosis; whereas *Ldh* reached its maximum level, which is approx. 7-fold of that at 0 h, within 2 h of anoxia. Enzyme activity studies (Fig. 13 and Fig. 14) reveal that both ADH and LDH activities increased as anaerobiosis developed in the aleurone layers; both of them reached their maximum levels at approximately 48 h of anoxia. Under various oxygen tensions (Fig. 14), ADH activity started to increase when oxygen concentration decreased to below 10%, and it continuously rose as oxygen tension decreased further to 0%. LDH activity reached its maximum level when oxygen concentration was decreased to 10%, and it then remained at the level as oxygen tension was decreased further.

4.3.4. Possible intracellular location of Hb in barley coleoptiles

Most Hbs are found in the cytoplasm (Wittenberg & Wittenberg, 1990). To investigate the possible intracellular locations of hemoglobin in barley, total soluble proteins together with mitochondrial soluble and mitochondrial membrane-bound proteins from coleoptiles were analysed using Western blotting. No detectable Hb was observed in any fraction of the un-induced coleoptiles (Fig. 15), whereas in induced coleoptiles, Hb was detected only in the total soluble protein fraction. No Hb bands were observed in mitochondrial fractions. The results indicate that Hb protein is mainly located in the cytoplasm.

Figure 13. Time course of ADH and LDH activities in barley aleurone layers subjected to anoxia. Enzyme was extracted from barley aleurone layers treated under anoxia (N_2) for various periods of time ranged from 0 to 72 h. Enzyme activity assay was carried out as described in "Materials and Methods". The data shown were the means ± SE values of 4 replicates.

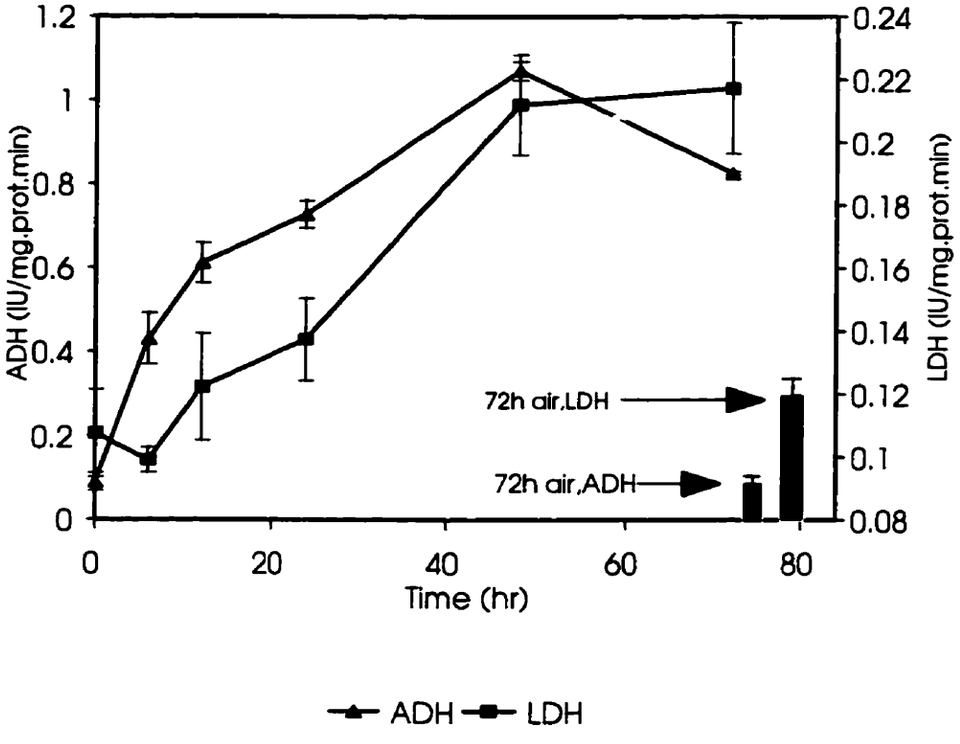

1.2
1
0.8
0.6
0.4
0.2
0
ADH (IU/mg.prot.min)
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
LDH (IU/mg.prot.min)
72h air,LDH
72h air,ADH
0
20
40
60
80
Time (hr)
ADH
LDH

Figure 14. ADH and LDH activities in barley aleurone layers subjected to various O_2 tensions. Crude enzyme activity from extracts of barley aleurone layers treated in various O_2 tensions from 0 to 20% for 24 h was analysed as described in "Materials and Methods". The data shown are the means ± SE values of 4 replicates.

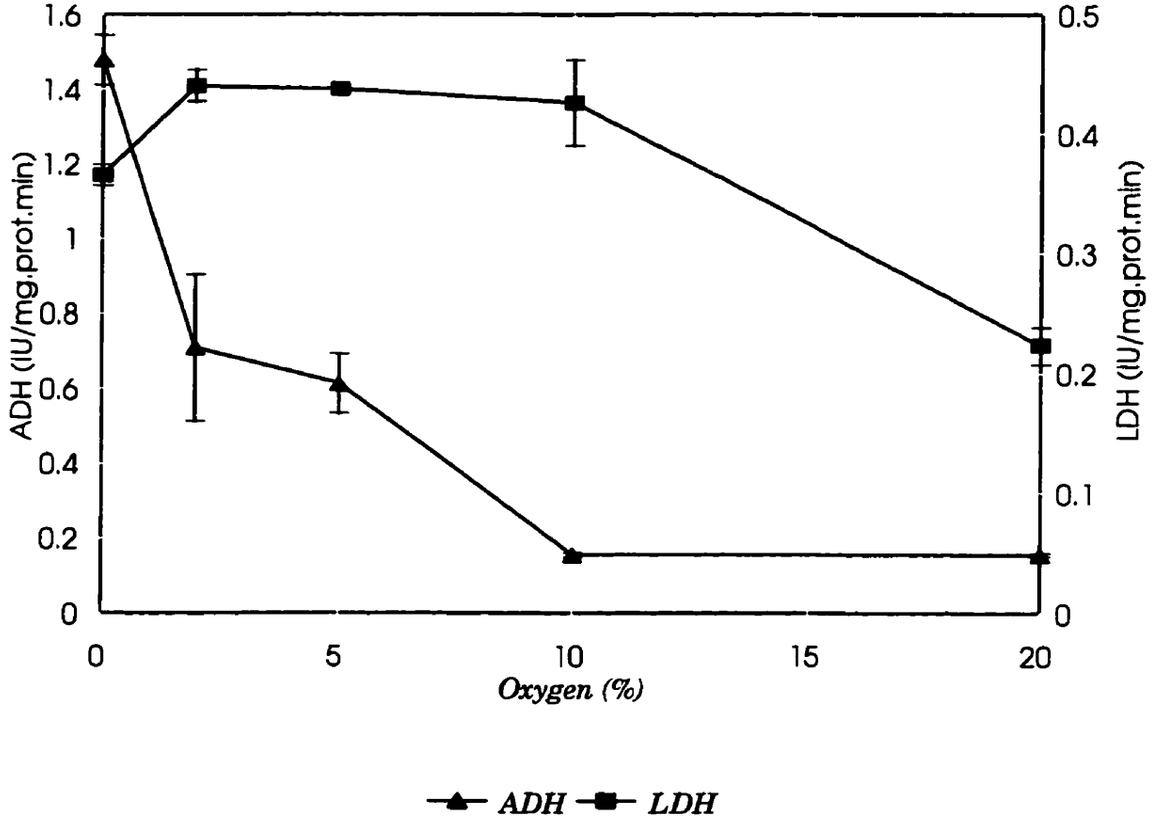
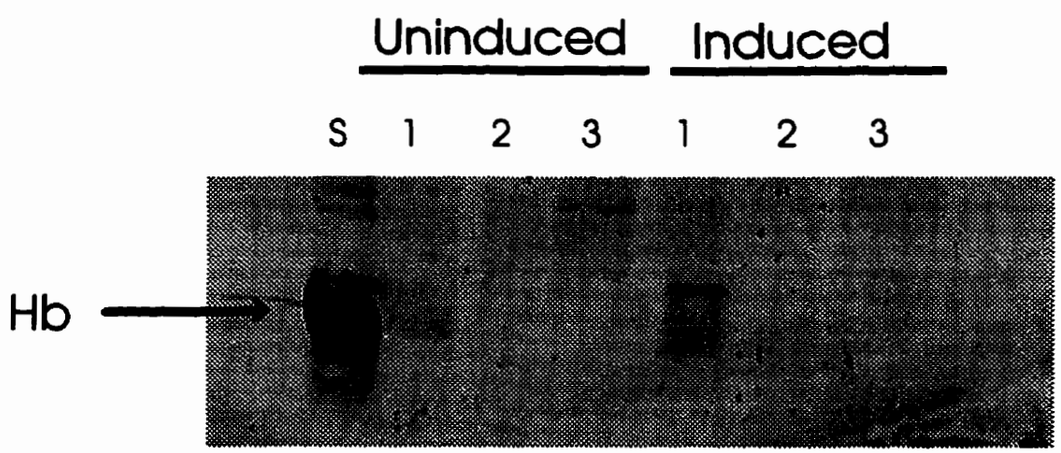

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ADH (IU/mg.prot.min)
0.5
0.4
0.3
0.2
0.1
0
LDH (IU/mg.prot.min)
0
5
10
15
20
Oxygen (%)
ADH
LDH

Figure 15. Intracellular location of Hb protein in barley coleoptiles. Different fractions of proteins were extracted from barley coleoptiles, Western blot analysis with Hb antibody was performed as described in "Materials and Methods". '*Uninduced*' and '*Induced*' represent the coleoptiles treated in air and N_2 for 24 h, respectively. '*s*' represents purified barley recombinant Hb. *1*- total soluble protein from the coleoptiles; *2*-mitochondrial soluble proteins; *3*-mitochondrial membrane-bound proteins.


Uninduced
Induced
S
1
2
3
1
2
3
Hb

4.4. Hb gene expression is related to respiration and ATP production

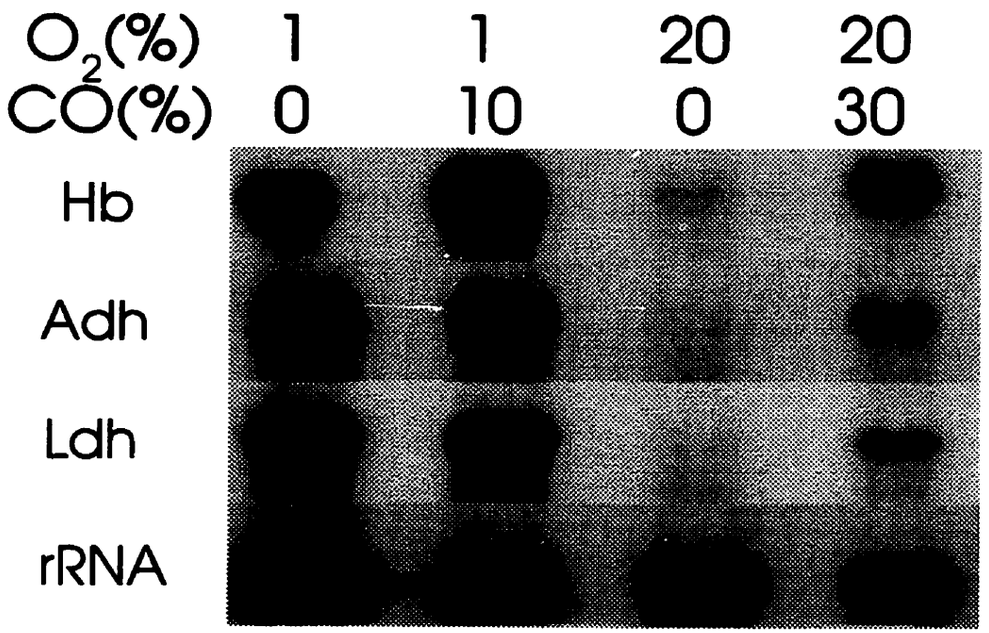
4.4.1. Hb gene expression in response to CO

The effect of CO on the transcription of Hb, as well as on ADH and LDH , two key enzymes involved in glycolysis, is shown in Fig. 16. All three genes were induced by hypoxia (1% O_2). CO strongly stimulated Hb transcription under both normoxic (20% O_2) and hypoxic (1% O_2) conditions. CO applied under normoxic conditions was more effective than hypoxic conditions at inducing Hb gene expression. The effect of CO on *Adh* and *Ldh* gene expression was less pronounced than its effect on Hb expression. CO increased both *Adh* and *Ldh* transcript levels under normoxia, but it was not as effective as hypoxia. Furthermore, 10% CO had only a slight effect on mRNA levels of the genes (*Adh* and *Ldh*) under hypoxic conditions.

4.4.2. Effects of cyanide and antimycin A on Hb gene expression

The conditions for CO treatment of aleurone layers were chosen to minimize the effects of CO on mitochondrial electron transport. To determine whether interference with mitochondrial electron transport affected hemoglobin gene expression, aleurone layers were incubated in the presence of either KCN or Antimycin A (AA) under both aerobic and anaerobic conditions (Fig. 17). KCN (0.8 mM) dramatically increased the level of Hb transcript under normal atmospheric conditions (Air atmosphere). KCN had no further inhibitory or stimulatory effect on anoxia-induced Hb transcript accumulation (Fig. 17a). Respiratory studies showed that KCN (0.8 mM) inhibited 25% of the total oxygen uptake, whether the respiration was measured 15 min or 6 h after KCN treatment (Table II).

Figure 16. Effect of carbon monoxide on Hb, Adh and Ldh gene expression. Freshly prepared barley aleurone layers were incubated in 1.5 mL incubation medium containing 10 μg/mL of chloramphenicol and 10 mM $CaCl_2$ and then treated with various gas mixtures for 24 h. Northern blot analysis was performed as described in 'Materials and Methods' and previous figures. The membrane was probed with barley Hb probe first, and then the membrane was stripped by boiling in 0.1% SDS for 5 min. The filter was reprobed with *Adh*1, and then with *Ldh*1 probe, and finally with rRNA probe. Signals from each membrane were quantified by an image analysis system. Messenger RNA signals were normalized to their corresponding 26S rRNA signals. Data represent the means ± SE values of two replicates per treatment.


O2(%) 1 1 20 20
CO(%) 0 10 0 30
Hb
Adh
Ldh
rRNA

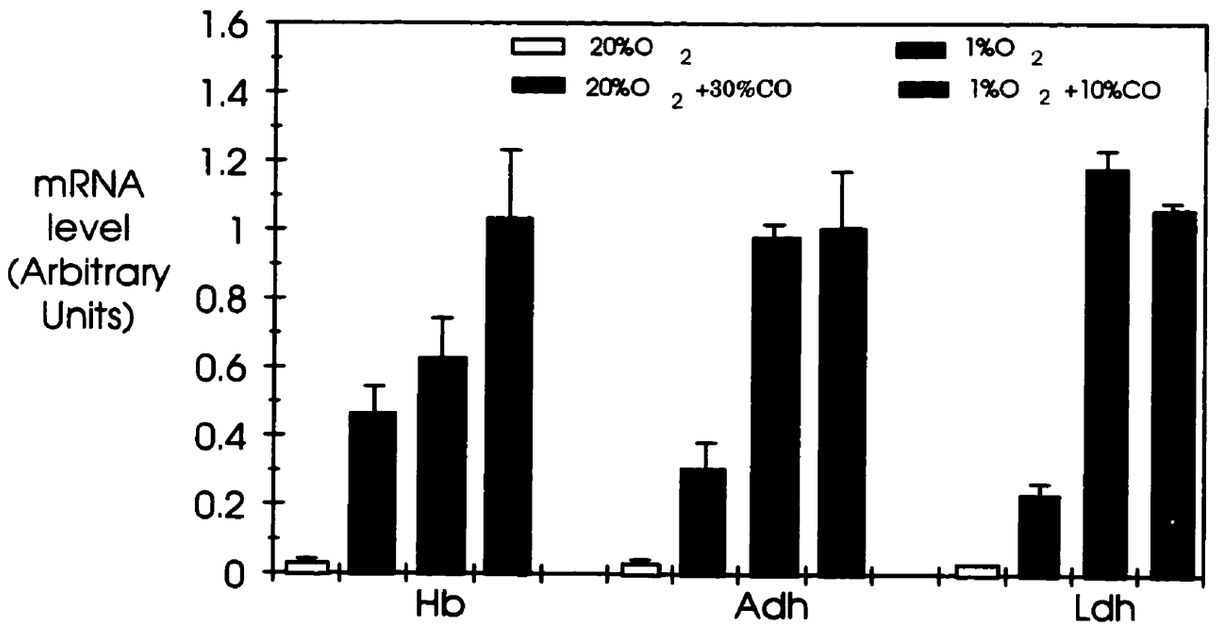
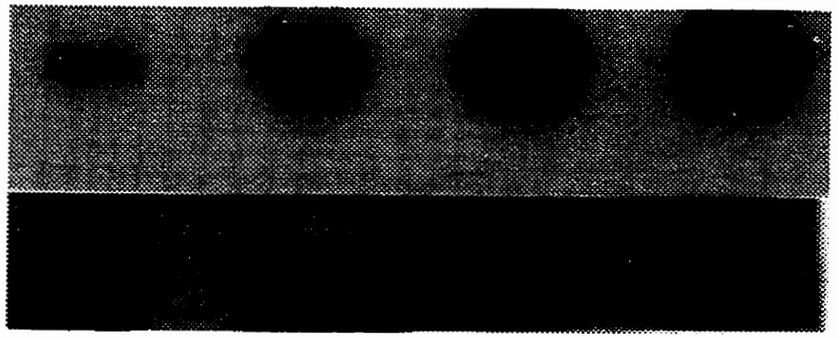
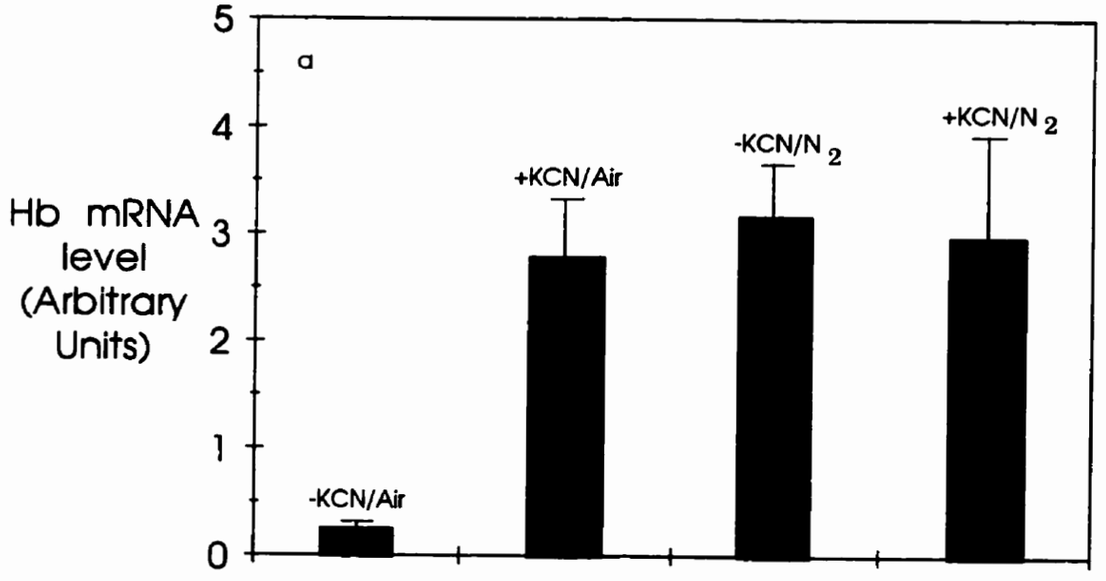

20%O 2
20%O 2 +30%CO
1%O 2
1%O 2 +10%CO
mRNA level (Arbitrary Units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Hb
Adh
Ldh

Figure 17. Effect of KCN and antimycin A on Hb transcription. *a*, KCN effect. Barley aleurone layers were preincubated in 0.1 M phosphate buffer (pH 7.0) with 0.8 mM KCN (+KCN) or without KCN (-KCN) for 1 h. The layers were then placed under anoxia (N_2) or air for 6 h in the same solutions. Northern blot analysis was carried out as described in "Materials and Methods". The blot was probed with the ^{32}P-labeled full-length barley Hb cDNA first, and then stripped and reprobed with the rDNA probe. Signals from two separate experiments of two blots each (totally 4 blots) were quantified by image analysis and normalized to the corresponding 26S rRNA signals. *b*. Antimycin A effect. Barley aleurone layers were preincubated in 0.2 mM antimycin A, diluted from 27 mM stock solution in 2-propanol, in 0.1 M phosphate buffer, pH 7.0 (+AA) or in same amount of 2-propanol as indicated in antimycin A solution in 0.1 M phosphate buffer, pH 7.0 (-AA) for 1 h. The layers were then placed under N_2 or air for 6 h in the same solutions. Northern blot analysis was carried out and presented the same as described for *a*.

a


Hb
rRNA


Hb mRNA level (Arbitrary Units)
5
4
3
2
1
0
a
-KCN/Air
+KCN/Air
-KCN/N 2
+KCN/N 2

b

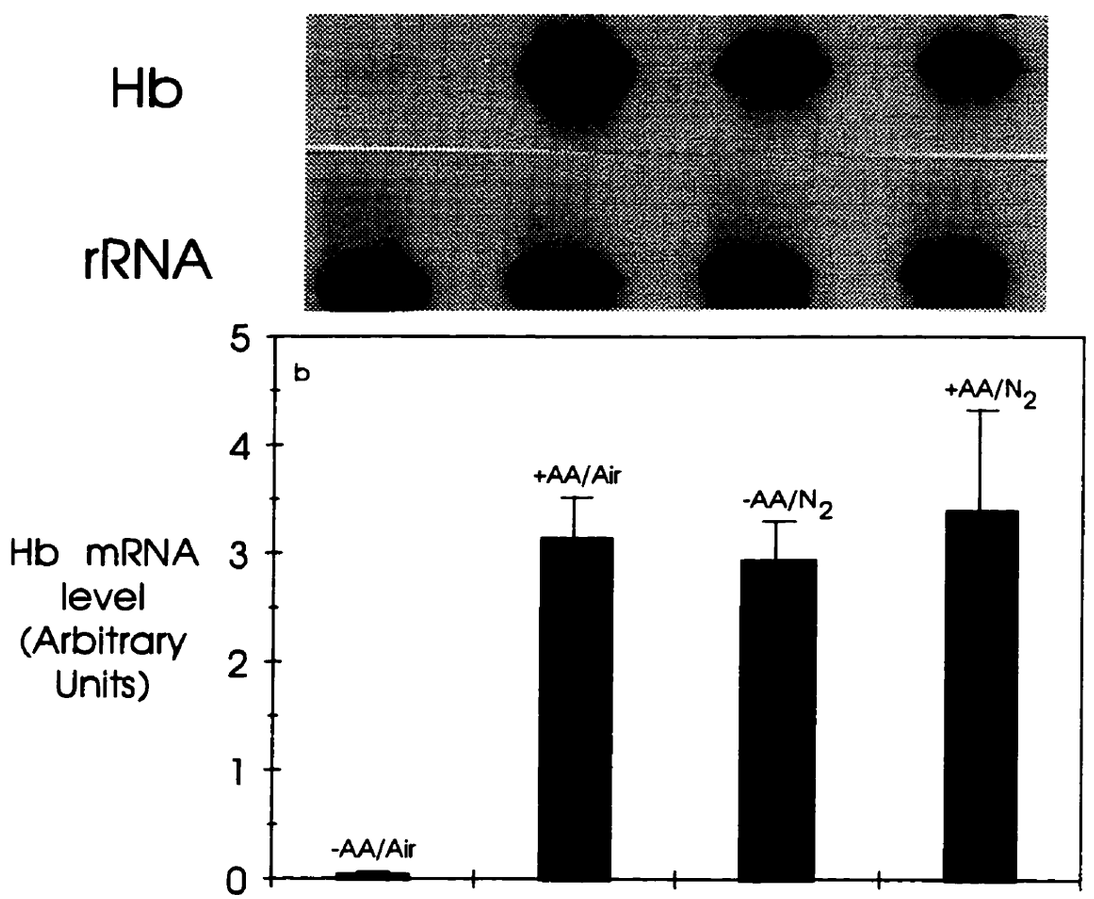

Hb
rRNA
b
5
4
3
2
1
0
Hb mRNA level (Arbitrary Units)
-AA/Air
+AA/Air
-AA/N2
+AA/N2

Table II. ***The effects of respiratory inhibitors on respiration in barley aleurone tissue***

Seven barley aleurone layers were set for oxygen consumption measurement at 25°C as described in the "Materials and Methods". The data shown are the means ± SE of two separate measurements. Control: 0.1 M phosphate buffer, pH 7.0; KCN: 0.8 mM in 0.1 M phosphate buffer, pH 7.0; antimycin A : 0.2 mM antimycin A, diluted from 27 mM stock solution in 2-propanol, in 0.1 M phosphate buffer, pH 7.0; 2-propanol: same amount of 2-propanol as indicated in antimycin A solution in 0.1 M phosphate buffer, pH 7.0; DNP: 0.1 mM in 0.1 M phosphate buffer, pH 7.0.

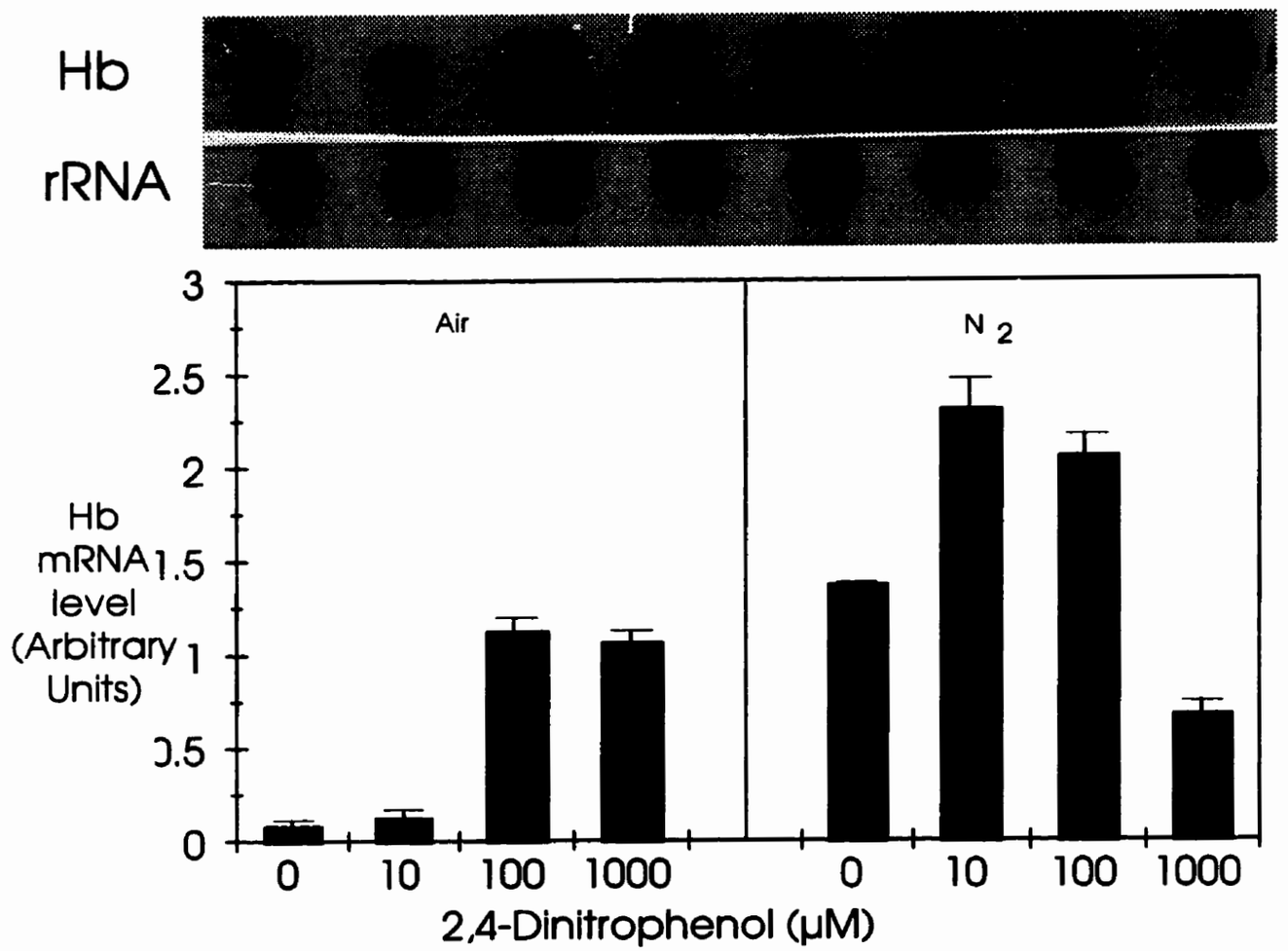
Treatment	15 min preincubation		6 h preincubation	
	$nmole.min^{-1}.layer^{-1}$	%	$nmole.min^{-1}.layer^{-1}$	%
Control	3.69 ± 0.11	100	2.95 ± 0.08	100
2-Propanol	3.81 ± 0.09	103.2	2.83 ± 0.06	95.9
Antimycin A	2.33 ± 0.03	63.1	2.09 ± 0.03	70.9
KCN	2.83 ± 0.06	76.7	2.09 ± 0.03	70.9
DNP	6.88 ± 0.20	186.5	4.00 ± 0.12	135.6

Antimycin A specifically interferes with the mitochondrial electron transport chain between cytochrome *b* and cytochrome *c*. Respiratory studies demonstrated that antimycin A inhibited oxygen uptake of barley aleurone layers 40% and 30% when measured 15 min or 6 h respectively after treatment (Table II). Under aerated conditions, the level of Hb messenger RNA increased dramatically in aleurone layers treated with antimycin A (Fig. 17b). Under N_2, antimycin A had no further effect on Hb transcription.

4.4.3. Effects of 2,4-dinitrophenol on Hb gene expression

Inhibition of mitochondrial electron transport may induce Hb gene expression through several different routes. To gain further insight into how this occurred, other mitochondrial inhibitors were tested. 2,4-Dinitrophenol (DNP) depletes the chemosmotic potential developed across mitochondrial membranes during electron transport, resulting in increased electron transport without concomitant ATP production. Respiratory experiments indicated that 100 µM DNP stimulated oxygen uptake in barley aleurone layers by approximately 80% and 40%, measured 15 min and 6 h after treatment (Table II) respectively. Northern blot analysis (Fig. 18) showed that, under air, Hb transcripts did not change appreciably when aleurone layers were treated with 10 µM DNP. Messenger levels increased significantly, however, as the concentration of DNP in the incubation medium was increased from 10 to 100 µM. A concentration of 1 mM DNP gave roughly the same response as 100 µM DNP. Under anaerobic conditions, 10 µM DNP induced a greater Hb gene response than with DNP or anoxia alone. Concentrations of 100 µM DNP or higher, however, appeared to be less effective.

Figure 18. Effect of DNP on Hb transcription. Barley aleurone layers were preincubated in different 2,4-dinitrophenol (DNP) concentrations for 1 h. The layers were then placed under N_2 or air for another 6 h in the same solutions. Northern blot analysis was performed as described in the "Materials and Methods" with the ^{32}P-labeled barley Hb cDNA and then with the rDNA probe. Signals from the blots were quantified by image analysis and normalized to the corresponding 26S rRNA signals. Data are means ± SE values of 4 replicates.


Hb
rRNA
Air
N 2
Hb mRNA level (Arbitrary Units)
3
2.5
2
1.5
1
0.5
0
0
10
100
1000
0
10
100
1000
2,4-Dinitrophenol (μM)

4.4.4.Effects of oligomycin on Hb gene expression

Whereas DNP inhibits ATP synthesis by depletion of the chemosmotic potential, oligomycin inhibits by blocking phosphorylation of ADP which is driven by the chemosmotic gradient. The oxygen uptake of barley aleurone layers was not significantly affected by oligomycin in a range from 0 to 20 μg/mL (Table III). However, Northern blot analysis (Fig. 19) indicated that Hb transcript levels increased as the concentration of oligomycin increased from 0 to 5 μg/mL, under normal atmospheric conditions. At 5 μg/mL, the level of Hb transcripts was approximately the same as that obtained under anaerobic conditions in the absence of oligomycin. There was no further increase in Hb gene expression as the oligomycin concentration was increased to 20 μg/mL.

4.4.5. Effects of BHAM, an alternative oxidase inhibitor, on Hb gene expression

Alternative oxidase is characterized by its resistance to cyanide and antimycin A (McIntosh, 1994). Thus, in the presence of KCN or antimycin A, the cytochrome *c* pathway is no longer functional, and alternative electron transport to oxygen through the alternative oxidase becomes the major pathway. However, since this pathway skips complexes III and IV, ATP production coupled with electron transport is dramatically reduced. To investigate the possible involvement of an alternative pathway in Hb gene expression, benzhydroxamic acid (BHAM) was used. Northern blotting analysis indicates that 2 mM BHAM slightly induced Hb transcription (Fig. 20), whereas KCN had much stronger effect. KCN combined with BHAM had a very similar effect as KCN alone. Oxygen consumption of aleurone layers was reduced by BHAM and KCN (Table IV): approximately 27% of oxygen uptake was inhibited by KCN or 2 mM BHAM alone, whereas approximately 73% was inhibited by the KCN and 2 mM BHAM combined.

Table III. ***The effects of oligomycin on barley aleurone tissue respiration***

Seven barley aleurone layers were set for oxygen consumption measurement at 25 °C as described in the "Materials and Methods". The data shown are means ± SE values of two or three separate measurements. Each concentration was diluted from 1000 µg/mL stock solution in 2-propanol. All solutions contained equal amount of 2-propanol in 0.1 M phosphate buffer, pH 7.0.

Oligomycin (µg/mL)	Oxygen uptake	
	O_2 nmole/layer.min	%
0	2.01±0.00	100
0.625	1.88±0.27	93.5
1.25	1.95±0.09	97.0
2.5	1.85±0.06	92.0
5	1.85±0.06	92.0
10	1.90±0.11	94.5
20	1.91±0.11	95.0

Figure 19. Effect of oligomycin on Hb transcription. Barley aleurone layers were preincubated in different concentrations for 1 h. The layers were then placed under N_2 or air for another 6 h in the same solutions. Total RNA was isolated. Ten micrograms of total RNA were electrophoresed in denaturing 1.25% agarose gel containing a trace (5 mg/L) of ethidium bromide. Northern blot analysis was carried out with the barely Hb and then rDNA probes as described in the "Materials and Methods". Hb mRNA signals were quantified and normalized as described in previous figures. The data are means $\pm$ SE values of three replicates.

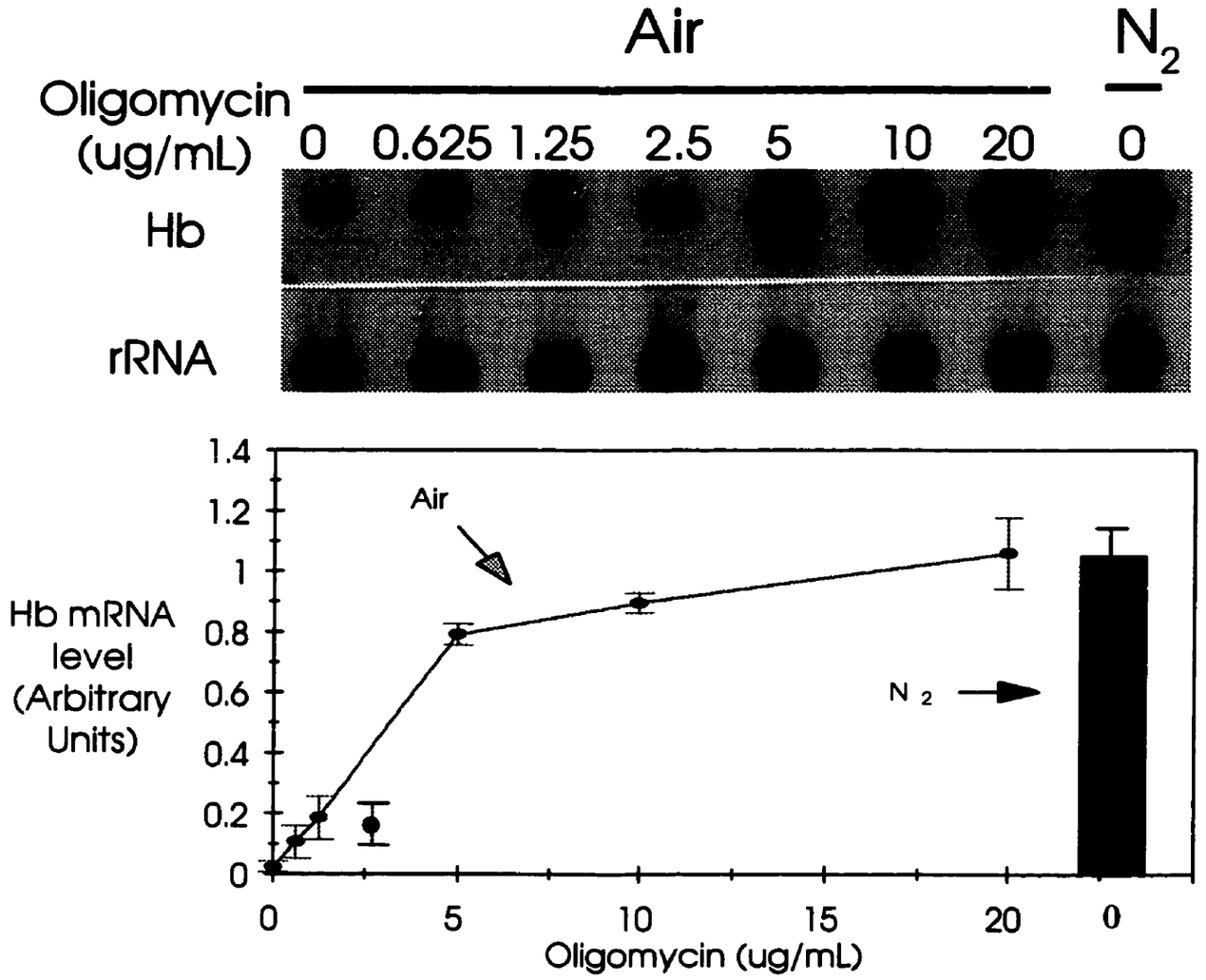

Air
N2
Oligomycin (ug/mL)
0 0.625 1.25 2.5 5 10 20 0
Hb
rRNA
Hb mRNA level (Arbitrary Units)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Air
N 2
0 5 10 15 20 0
Oligomycin (ug/mL)

Figure 20. Effect of benzhydroxamic acid (BHAM) on Hb gene expression. Barley aleurone layers were preincubated in 0.1 M phosphate buffer (pH 7.0) containing BHAM and/or KCN for 1 h. The layers were then placed under N_2 or air for another 6 h. Total RNA was isolated, and Northern blot analysis with the barley Hb cDNA probe was performed as described in the "Materials and Methods". 'ck' represents the treatment without inhibitors (i.e., buffer alone); BHAM was used at a concentration of 2.0 mM, KCN was used at a concentration of 0.8 mM, BHAM/KCN represents the treatment with both inhibitors (2.0 mM BHAM and 0.8 mM KCN). The numbers shown below the blot are intensities and areas of the corresponding bands, obtained by image analysis of the blots. RNA gel stained with ethidium bromide is shown to indicate the loading equivalence and RNA integrity.

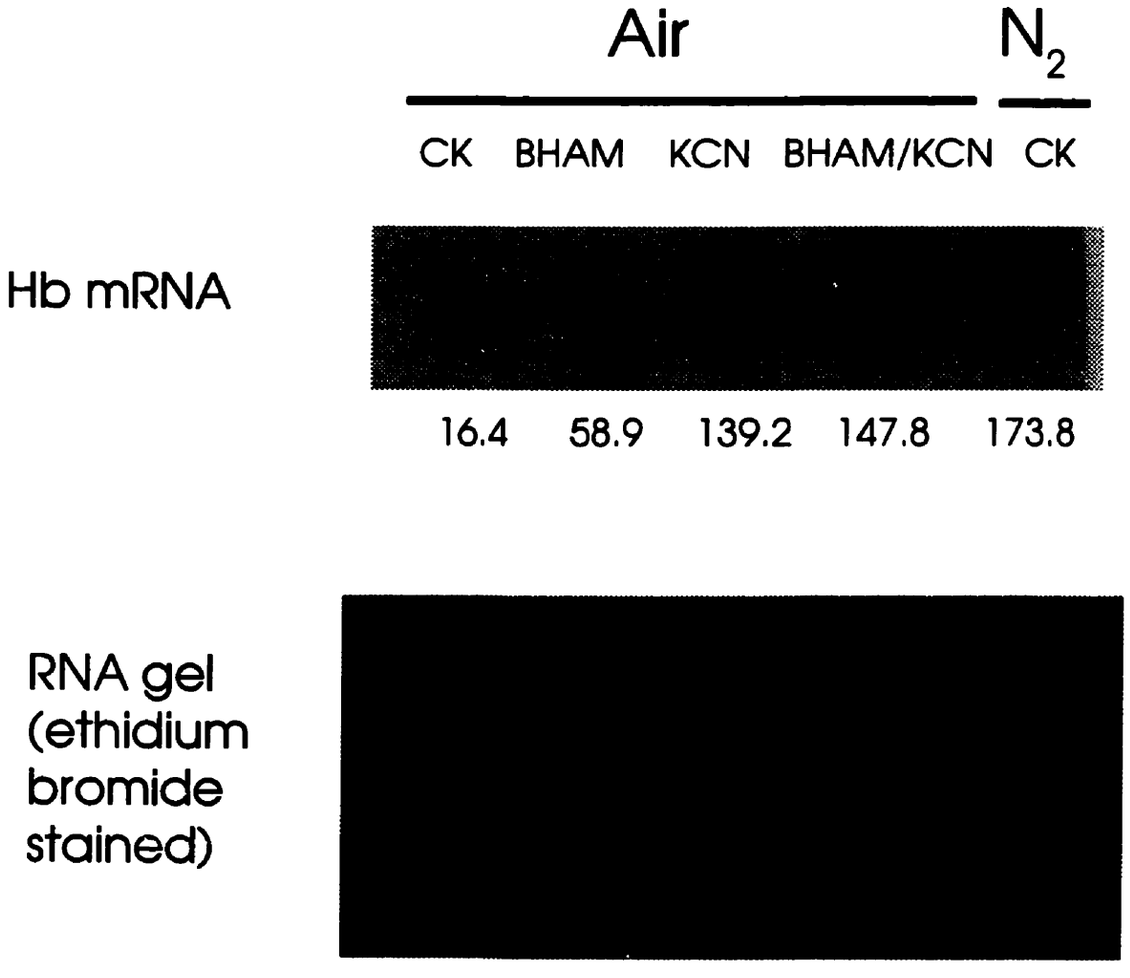

Air
N_2
CK
BHAM
KCN
BHAM/KCN
CK
Hb mRNA
16.4
58.9
139.2
147.8
173.8
RNA gel
(ethidium
bromide
stained)

Table IV. ***The effects of KCN and benzhydroxamic acid (BHAM) on oxygen consumption in barley aleurone layers***

Seven barley aleurone layers were set for oxygen consumption measurement at 25°C as described in the "Materials and Methods". The data shown are means ± SE values of two or three separate measurements. All solutions were buffered with 0.1 M phosphate buffer (pH 7.0).

Treatment	Oxygen uptake	
	O_2 nmole/layer.min	%
Buffer alone	3.77 ± 0.19	100
KCN (0.8 mM)	2.76 ± 0.03	73.2
0.8 mM BHAM	3.30 ± 0.04	87.5
2 mM BHAM	2.74 ± 0.17	72.7
0.8 mM BHAM+KCN	1.40 ± 0.04	37.1
2 mM BHAM+KCN	1.01 ± 0.03	26.8

4.4.6. Effect of nitrate on Hb gene expression

While nitrate has no specific effect on mitochondrial respiration in plants, there is a considerable requirement for reducing equivalents in the conversion of nitrate to ammonia. Furthermore, nitrate reductase is induced under oxygen deficiency in roots. The effect of nitrate on barley aleurone Hb gene expression was determined (Fig. 21). Hb transcript levels increased slightly when the concentration of nitrate increased from 0 to 10 mM under aerobic conditions, then declined as the concentration of nitrate was increased to 100 mM. Under anaerobic conditions there was an approximately 40% increase in Hb gene expression in the presence of 1 mM and 10 mM nitrate, but the addition of 100 mM nitrate inhibited Hb gene expression induced by the anaerobic conditions.

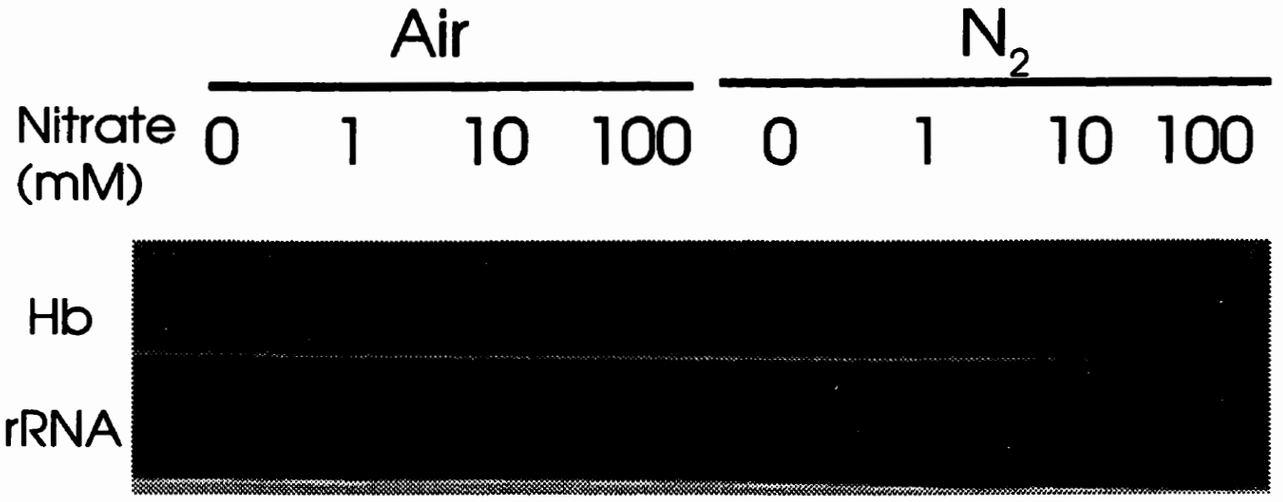
4.5. Signal transduction for Hb gene expression

4.5.1. Possible involvement of Ca^{2+} in Hb gene expression

4.5.1.1. Effects of ruthenium red (RR) on Hb and *Adh* gene expression

Ca^{2+} is suggested to act as a second messenger in many processes in organisms (Bowler & Chua, 1994). It is believed that Ca^{2+} is mainly stored in the rough ER (Denton *et al.*, 1980) or vacuole (Trewavas & Knight, 1994). Ruthenium red modifies Ca^{2+} release by blocking intercellular Ca^{2+} channels (Trewavas & Knight, 1994). From Fig. 22, it can be seen that under anoxic conditions, as the concentration of RR applied was increased, Hb transcript levels decreased. A similar pattern was found in *Adh* mRNA. These results suggested that Hb as well as *Adh* gene expression is calcium influx ($[Ca^{2+}]_i$)-dependent. However, unlike that previously reported in maize roots (Subbaiah *et al.*, 1994a), exogenously added Ca^{2+} failed to restore the

Figure 21. Effects of nitrate on Hb gene expression. Barley aleurone layers were preincubated in 1.5 mL incubation medium containing 10 mg/mL chloramphenicol and various concentrations of KNO_3 for 1 h as described in the "Materials and Methods". The layers were then placed under N_2 or air for another 24 h in the same solutions. Total RNA was isolated. Northern blot analysis was carried out and presented as described in the "Materials and Methods" and previous figures. The data shown were means ± SE values of 3 replicates.


Air
N2
Nitrate (mM)
0
1
10
100
0
1
10
100
Hb
rRNA

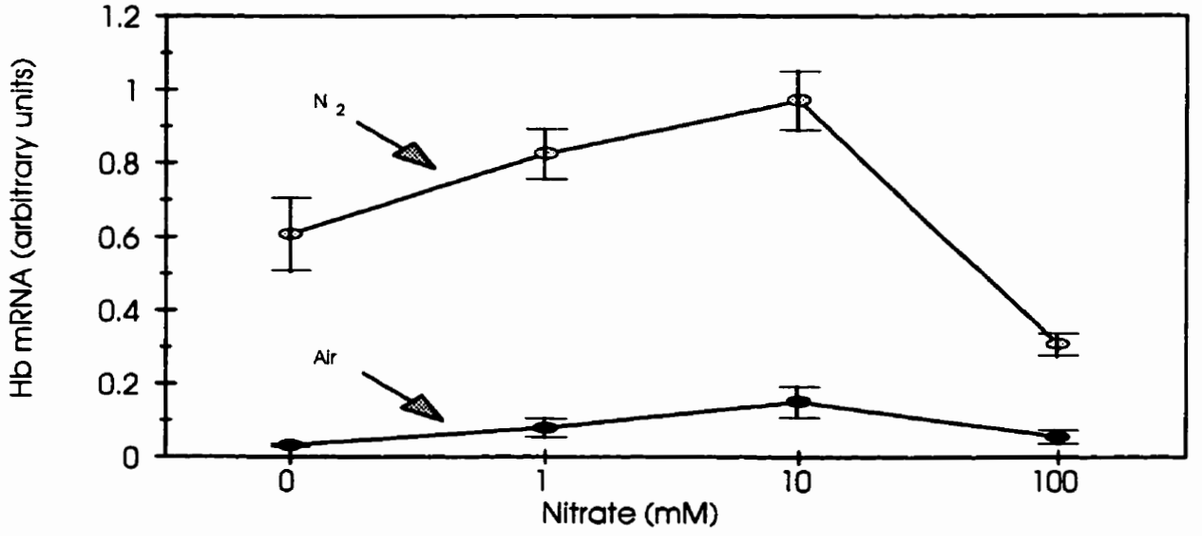
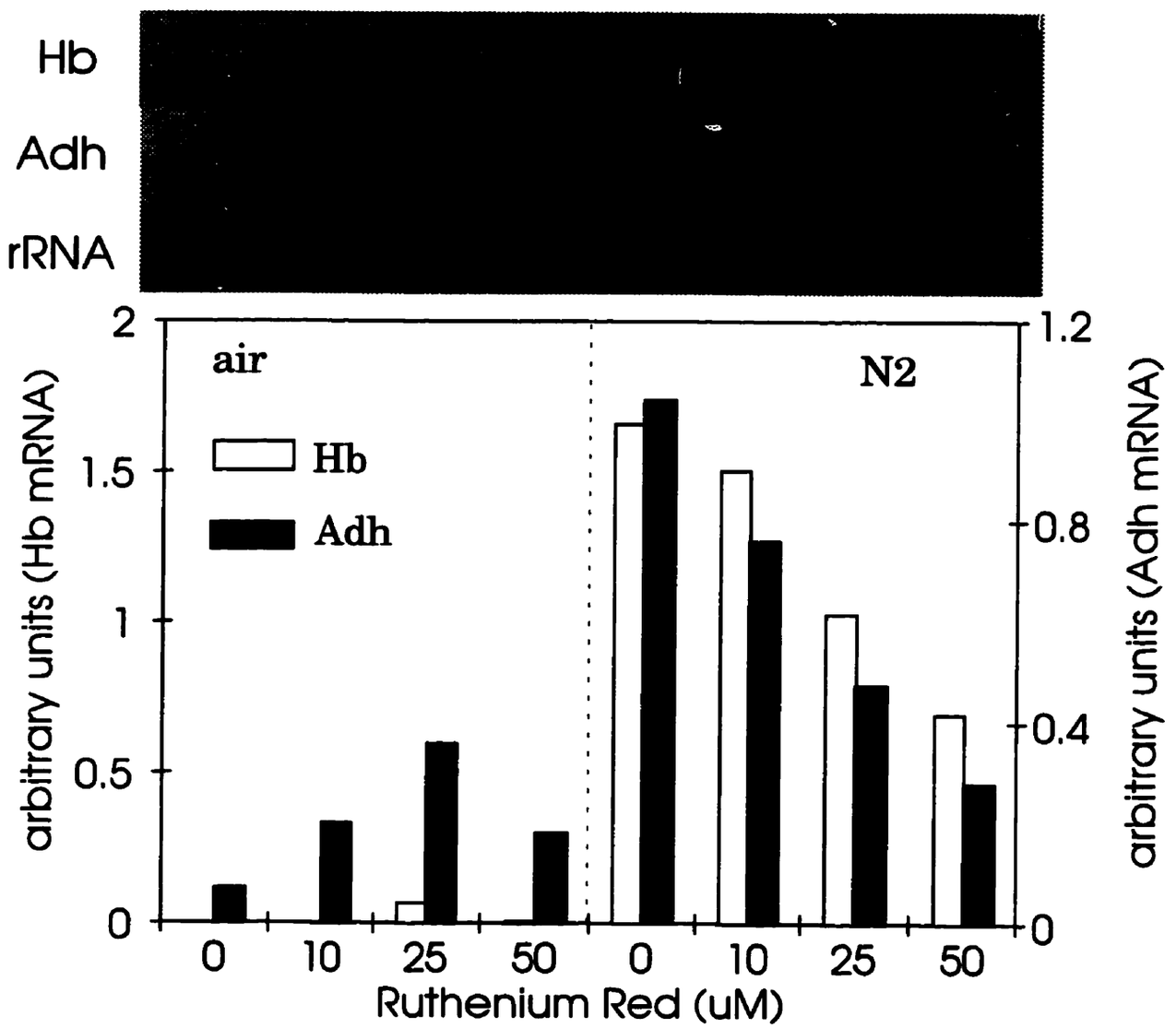

Hb mRNA (arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
N 2
Air
0
1
10
100
Nitrate (mM)

Figure 22. Effect of ruthenium red on Hb and Adh gene expression. Barley aleurone layers were preincubated in different concentrations of ruthenium red (RR) for 1 h. The layers were then placed under anoxia (N_2) or air for 6 h. Total RNA was isolated. Northern blot analysis was carried out as described in the "Materials and Methods". The filter was probed with the barley Hb probe first. After being stripped by boiling in 0.1% SDS for 5 min, the membrane was reprobed with a *Adh*1 probe, and finally with a rDNA probe. Signals from each membrane were quantified by image analysis. Messenger RNA signals were normalized to their corresponding 26S rRNA signals.


Hb
Adh
rRNA
air
N2
Hb
Adh
arbitrary units (Hb mRNA)
arbitrary units (Adh mRNA)
2
1.5
1
0.5
0
1.2
0.8
0.4
0
0
10
25
50
0
10
25
50
Ruthenium Red (uM)

normal responses of Hb and *Adh* to anoxia in barley aleurone layers after RR was removed from the incubation medium (data not shown). Interestingly, under aerobic conditions, increased concentration of RR induced *Adh* transcription. A slight stimulation was also observed in Hb transcription.

4.5.1.2. Effects of EGTA and A23187 on Hb and *Adh* gene expression

To gain more information about possible involvement of calcium in Hb as well as *Adh* gene regulation in barley aleurone layers, a divalent ionophore, A23187, and a metal chelator, EGTA, were used in the study. Under anaerobic conditions (N_2 atmosphere), A23187 slightly decreased Hb mRNA levels. EGTA reduced the messenger levels by 2- to 3-fold. A23187 combined with EGTA significantly decreased Hb mRNA levels (Fig.23). A very similar pattern was found in *Adh* transcripts. Under aerobic conditions, A23187 and A23187 combined with EGTA appeared to slightly increase Hb transcript level.

Since EGTA and A23187 may affect all free divalent metal ions, especially Ca^{2+} and Mg^{2+}, in cells, exogenous Ca^{2+} and Mg^{2+} were added to the barley aleurone layers after carefully and completely (as much as possible) removing EGTA and A23187 from the incubation medium in order to observe the responses of Hb and *Adh* to anoxia in the tissue. Under anaerobic conditions (Fig. 24), as expected, both Hb and *Adh* mRNA levels in the tissue were significantly decreased by the treatment of EGTA combined with A23187. Pronounced high levels of Hb and *Adh* transcripts were observed in the treatment in which exogenous calcium was added after removing EGTA and A23187; while in the treatment in which exogenous Mg^{2+} was added after removing EGTA and A23187, no significant changes were noticed. The results indicate that calcium, not magnesium, is involved in both *Adh* and Hb gene regulation.

Figure 23. Effects of EGTA and A23187 on Hb and Adh gene expression. Barley aleurone layers were preincubated in various additions at the concentrations described in "Materials and Methods". The layers were then placed under N_2 or air for 6 h. Total RNA was isolated. Northern blot analysis was carried out subsequently. The blot was probed with the Hb probe first, and then stripped and reprobed with the *Adh*1, and finally with the rDNA probe as described in previous figures and "Materials and Methods". Signals from the blots were quantified and normalized to the corresponding 26S rRNA. The data for Hb and *Adh* are presented as means ± SE (Hb) or means (*Adh*) of three replicates. 'E+A' represents EGTA plus A23187.

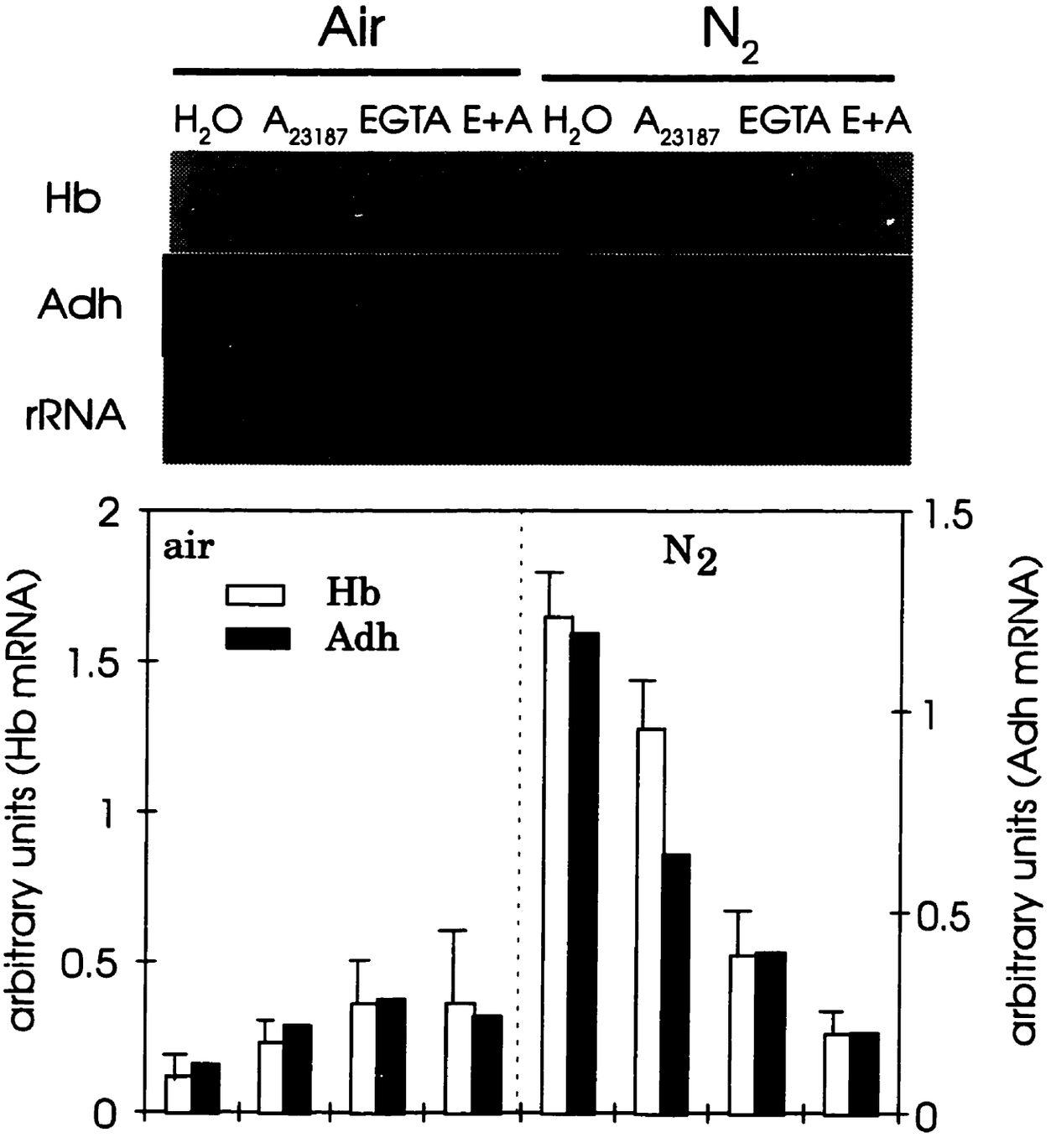
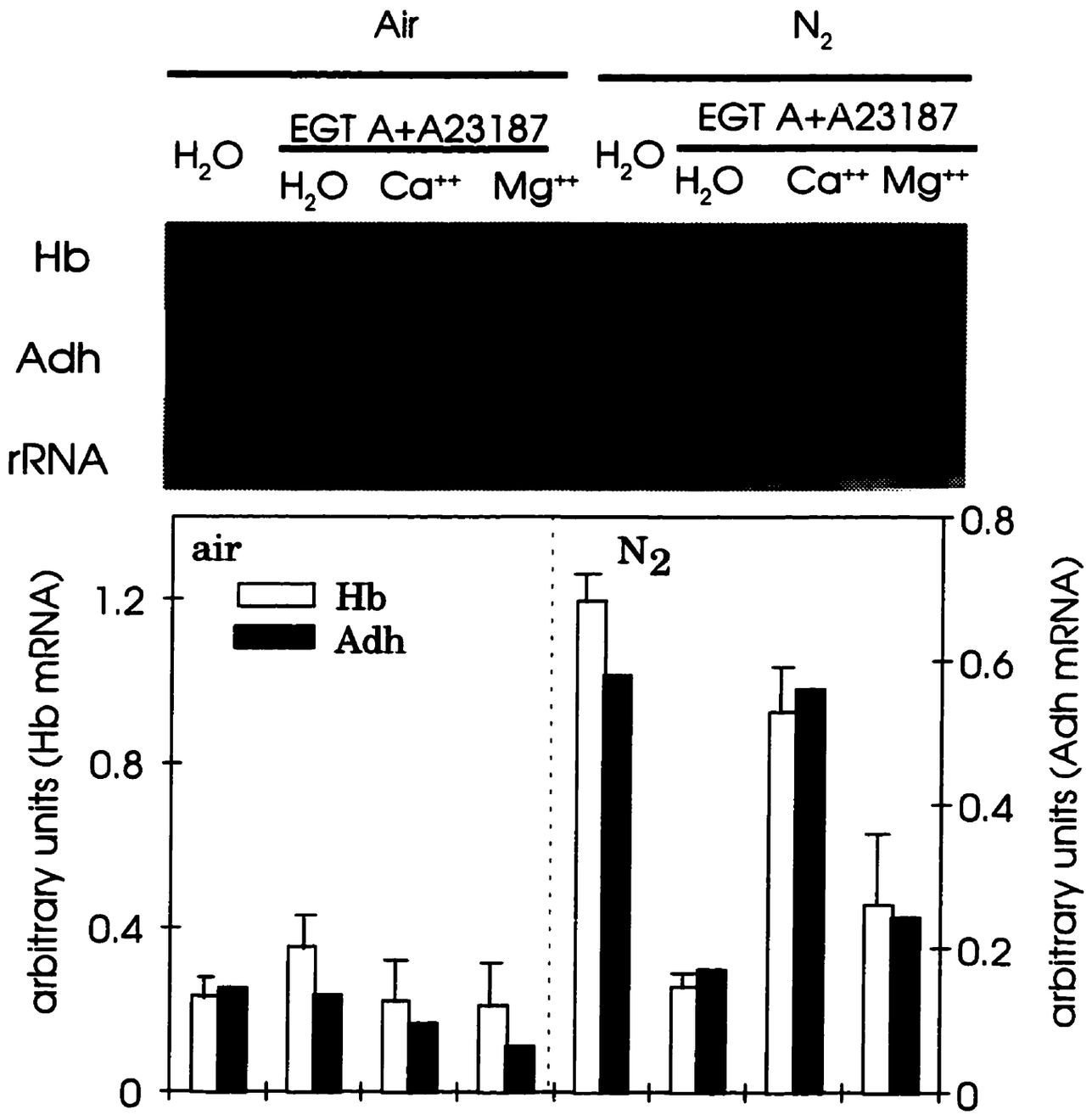

Air
N2
H2O A23187 EGTA E+A H2O A23187 EGTA E+A
Hb
Adh
rRNA
air
N2
Hb
Adh
arbitrary units (Hb mRNA)
arbitrary units (Adh mRNA)
2
1.5
1
0.5
0
1.5
1
0.5
0

Figure 24. Effects of Ca^{2+} and Mg^{2+} on Hb gene expression. Barley aleurone laycrs were preincubated in water or EGTA plus A23187 for 2 h as described in the "Materials and Methods". The layers incubated in EGTA+A23187 were washed carefully with sterilized-double-distilled water several times in order to remove remaining residues of EGTA and A23187. The layers were then incubated with water or $CaCl_2$ (1 mM) or $MgCl_2$ (5 mM) for 1 h. Thereafter, the layers were placed under N_2 or air for another 6 h in the same solutions. Total RNA was isolated and Northern blot analysis was carried out and presented as described in "Materials and Methods" and the Figure 23. The data for Hb and *Adh* are presented as means $\pm$ SE (Hb) or means (*Adh*) of three replicates.


Air
N2
H2O
EGT A+A23187
H2O Ca++ Mg++
H2O
EGT A+A23187
H2O Ca++ Mg++
Hb
Adh
rRNA
air
N2
Hb
Adh
arbitrary units (Hb mRNA)
arbitrary units (Adh mRNA)
1.2
0.8
0.4
0
0.8
0.6
0.4
0.2
0

4.5.1.3. Effects of W-7, a CaM antagonist, on Hb gene expression

Ca^{2+}-dependent calmodulin is involved in many calcium-mediated processes (Bowler & Chua, 1994). W-7, a calmodulin antagonist, was used in this study to investigate the possible involvement of CaM in Hb and *Adh* gene regulation in barley aleurone tissue. Fig. 25 shows the effect of W-7 at different concentrations on Hb and *Adh* transcription. Under anaerobic conditions, no significant changes in Hb and *Adh* mRNA levels were observed in the treatments in which W-7 concentration was less than 100 μM. However, as the concentration of W-7 increased to 1000 μM, the levels of both transcripts decreased. Interestingly, under aerated conditions, it appeared that both transcripts were enhanced by W-7. However, the extent of induction by W-7 under aerobic conditions was much less than that caused by oxygen deprivation.

4.5.2. Do G-proteins play a role in Hb gene expression? Effect of cholera toxin on Hb gene expression in barley aleurone tissue and aleurone protoplasts

Cholera toxin is a protein complex composed of two subunits, A and B. Subunit B binds to the outer membrane of the cell and then subunit A penetrates the membrane, binds to the Gα of subunit G-proteins and thus locks the G-proteins into their active form (see Voet & Voet, 1990). Cholera toxin was applied in both barley aleurone tissue and aleurone protoplasts in this study. No notable changes in Hb transcript levels were observed in the presence or absence of cholera toxin in either protoplasts or aleurone cells (Fig. 26). Anaerobic treatment of aleurone protoplasts failed to show any induction of Hb mRNA. This may be due to the fact that anaerobiosis had already developed during the imbibition period or because of unequal loading of RNA because of extremely low RNA yield. The results do not exclude possible involvement of G-protein in Hb gene regulation but provide no evidence in favour of this hypothesis.

Figure 25. Effects of W-7, a calmodulin antagonist, on Hb and Adh gene expression. Barley aleurone layers were preincubated in different concentrations of W-7 for 1 h. The layers were then placed under N_2 or air for another 6 h treatment in the same solutions. Total RNA was isolated. Northern blot analysis was carried out with the barley Hb probe, a *Adh* probe and a rDNA probe. The signals were quantified and normalized as described in the "Materials and Methods" and previous figures. The data shown are means $\pm$ SE values of 4 replicates.


Hb
Adh
rRNA

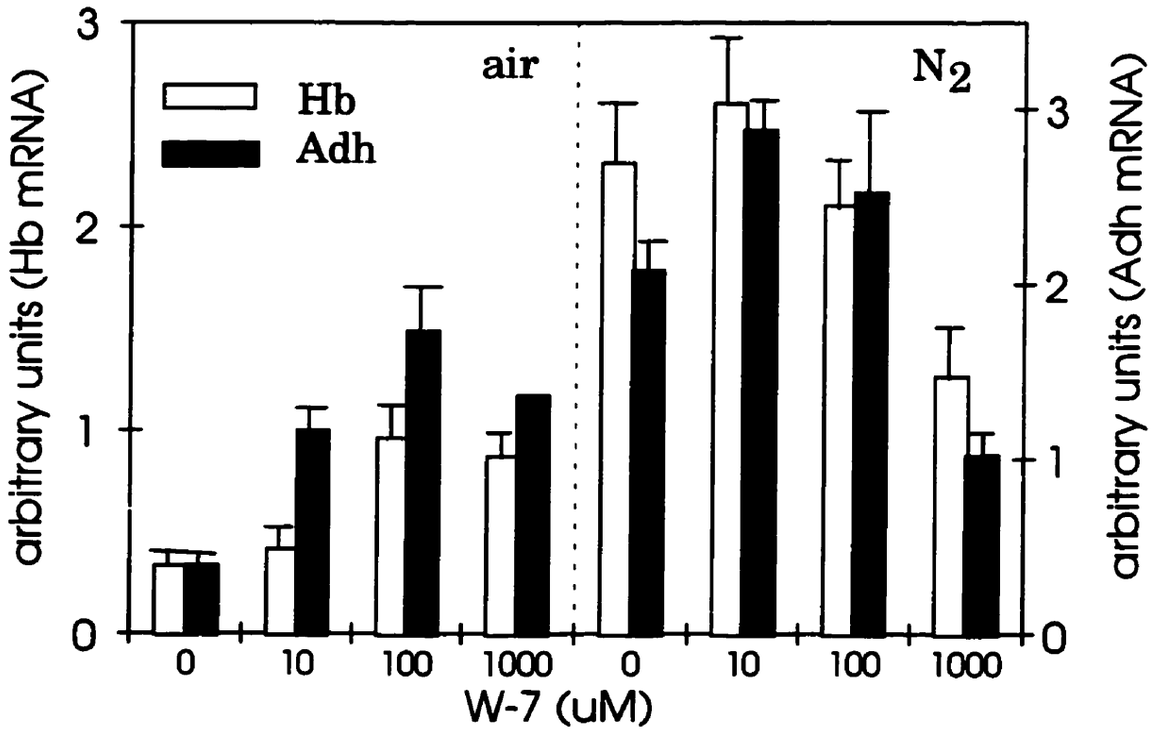
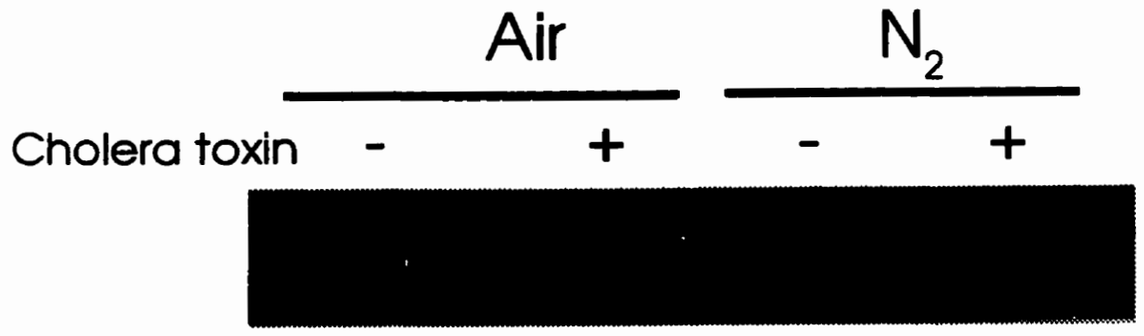

air
N2
Hb
Adh
arbitrary units (Hb mRNA)
arbitrary units (Adh mRNA)
0
1
2
3
0
10
100
1000
W-7 (uM)

Figure 26. Effect of cholera toxin on Hb gene expression in barley aleurone layers and barley aleurone protoplasts. a. Barley aleurone layers were preincubated in water (-) or cholera toxin (+) at the concentration of 1.5 µg/mL for 1 h. The layers were then placed under N_2 or air for another 24 h in the same solutions. Total RNA was isolated. Ten micrograms of total RNA was loaded and separated in the denaturing agarose gel (1.25%) by electrophoresis. After being blotted to Hybond -N^+, the RNA was probed with barley Hb cDNA probe. Loading equivalence was confirmed before the blotting as described in the "Materials and Methods". b. Barley aleurone protoplasts were isolated as described by Jacobsen et al. (1985). The protoplasts were incubated with or without 1.5 µg/mL cholera toxin for 6 h under anoxia or air. Total RNA was isolated. Northern blot analysis was carried out with the barley Hb probe as described above.

a. Aleurone layers



b. Aleurone protoplasts

4.5.3. Protein phosphorylation/dephosphorylation is involved in Hb gene expression

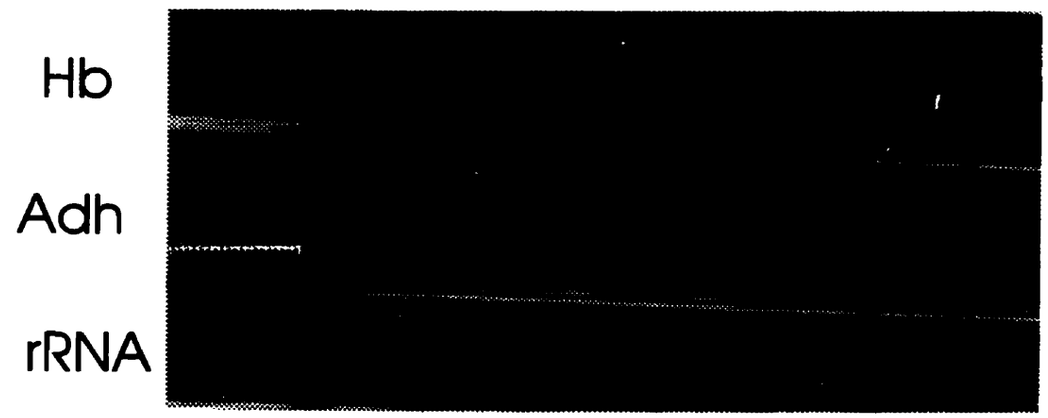
4.5.3.1. Effect of A3, a protein kinase inhibitor, on Hb gene expression

Reversible protein phosphorylation catalysed by protein kinases and protein phosphatases has been demonstrated to play a role in the regulation of many biological processes (Smith & Walter, 1996). A3, a protein kinase inhibitor, was applied at various concentrations in barley aleurone layers, and its effects on Hb and *Adh* gene expression were subsequently investigated. Under anaerobic conditions (Fig. 27), no significant changes were noticed in Hb mRNA levels at various concentrations of A3 from 0 to 200 μM. However, under the same conditions, as the concentration of A3 increased to 100 μM , about 2-fold decrease in *Adh* mRNA levels was observed. As the concentration was increased to 200 μM, an approximately 3- to 4-fold decrease in *Adh* transcription was seen. Under aerated conditions, both *Adh* and Hb transcriptions were enhanced by 10 and 100 μM concentrations of A3.

4.5.3.2. Effect of okadaic acid, a protein phosphatase inhibitor, on Hb gene expression

Okadaic acid (OA) inhibits protein phosphatase type 1 and 2A (Smith & Walter, 1996). A series of concentrations ranged from 0 to 200 nM of OA were used to treat barley aleurone layers, and the effects of OA on Hb and *Adh* gene expression were studied. Anoxia-induced Hb and *Adh* transcription was strongly inhibited by okadaic acid (Fig. 28). Under anoxic conditions, as the concentration of OA was increased from 0 to 10 nM, about 30% and 60% reduction of Hb and *Adh* transcripts, respectively, was observed (Fig. 28). As the concentration was increased further to 100 nM and finally to 200 nM, more significant reduction in both Hb and *Adh* mRNA was observed. The results indicate that okadaic acid-sensitive protein phosphatase is involved in

Figure 27. Effects of A3, a protein kinase inhibitor, on Hb and Adh gene expression. Freshly prepared barley aleurone layers were preincubated in various concentrations of A3 for 1 h. The layers were then placed under N_2 or air for another 6 h in the same solutions. Total RNA was isolated, and Northern blot analysis was performed with a barley Hb probe, then a *Adh* probe and finally a rDNA probe as described in the "Materials and Methods". The signals from the blots were quantified and normalized as described in the previous figures. The data shown are means $\pm$ SE of four replicates.


Hb
Adh
rRNA

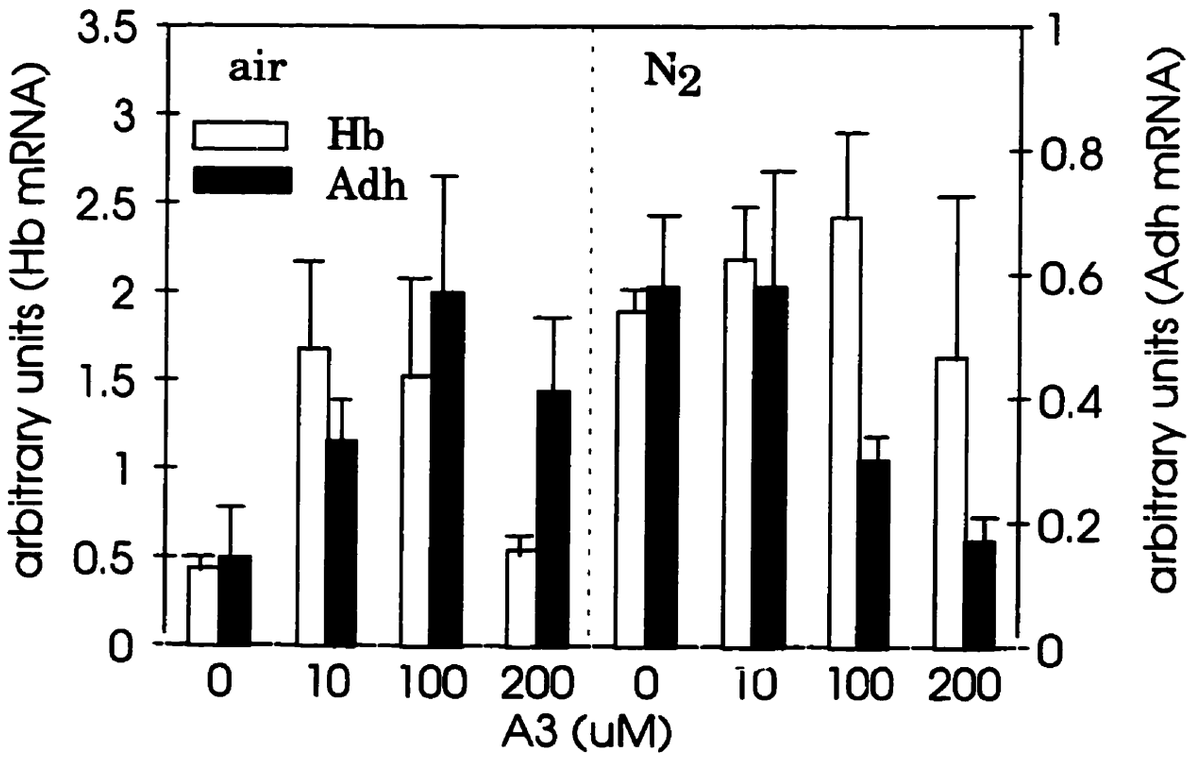
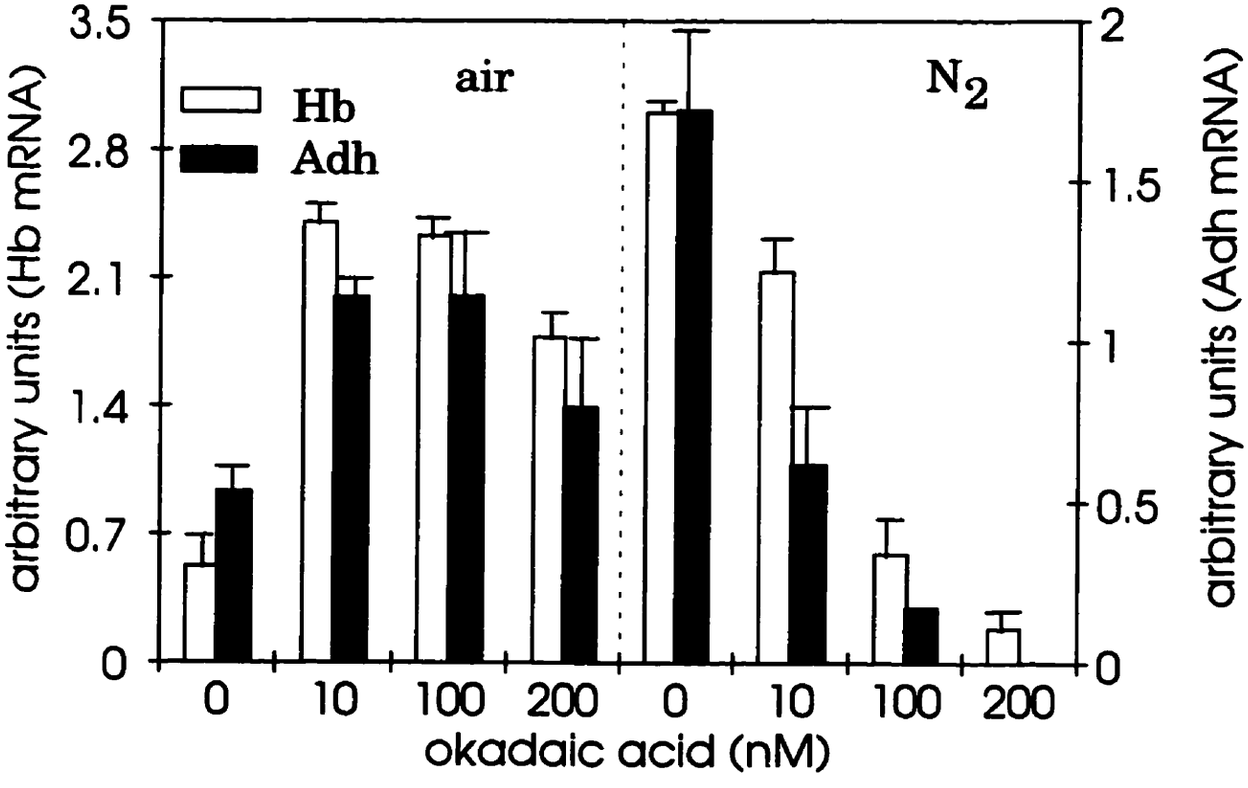

air
N2
Hb
Adh
arbitrary units (Hb mRNA)
arbitrary units (Adh mRNA)
3.5
3
2.5
2
1.5
1
0.5
0
1
0.8
0.6
0.4
0.2
0
0
10
100
200
0
10
100
200
A3 (uM)

Figure 28. Effects of okadaic acid on Hb and Adh gene expression. Freshly prepared barley aleurone layers were preincubated in various concentrations of okadaic acid (OA) for 1 h. The layers were then incubated in N_2 or air for another 6 h in the same solutions. Total RNA was isolated, and Northern blot analysis was carried out with a barley Hb probe, then a Adh probe and finally a rDNA probe as described in the "Materials and Methods". The signals from the blots were quantified and normalized as described in previous figures. The data shown blow the blots are means $\pm$ SE values of four replicates.


Hb
Adh
rRNA


3.5
2.8
2.1
1.4
0.7
0
arbitrary units (Hb mRNA)
air
N2
Hb
Adh
2
1.5
1
0.5
0
arbitrary units (Adh mRNA)
0
10
100
200
0
10
100
200
okadaic acid (nM)

anaerobic-induced Hb and *Adh* gene expression. However, under normoxia, it appeared that both transcripts were induced by OA in the range 10 to 200 nM.

4.5.3.3. Effect of cycloheximide, a protein synthesis inhibitor, on Hb gene expression

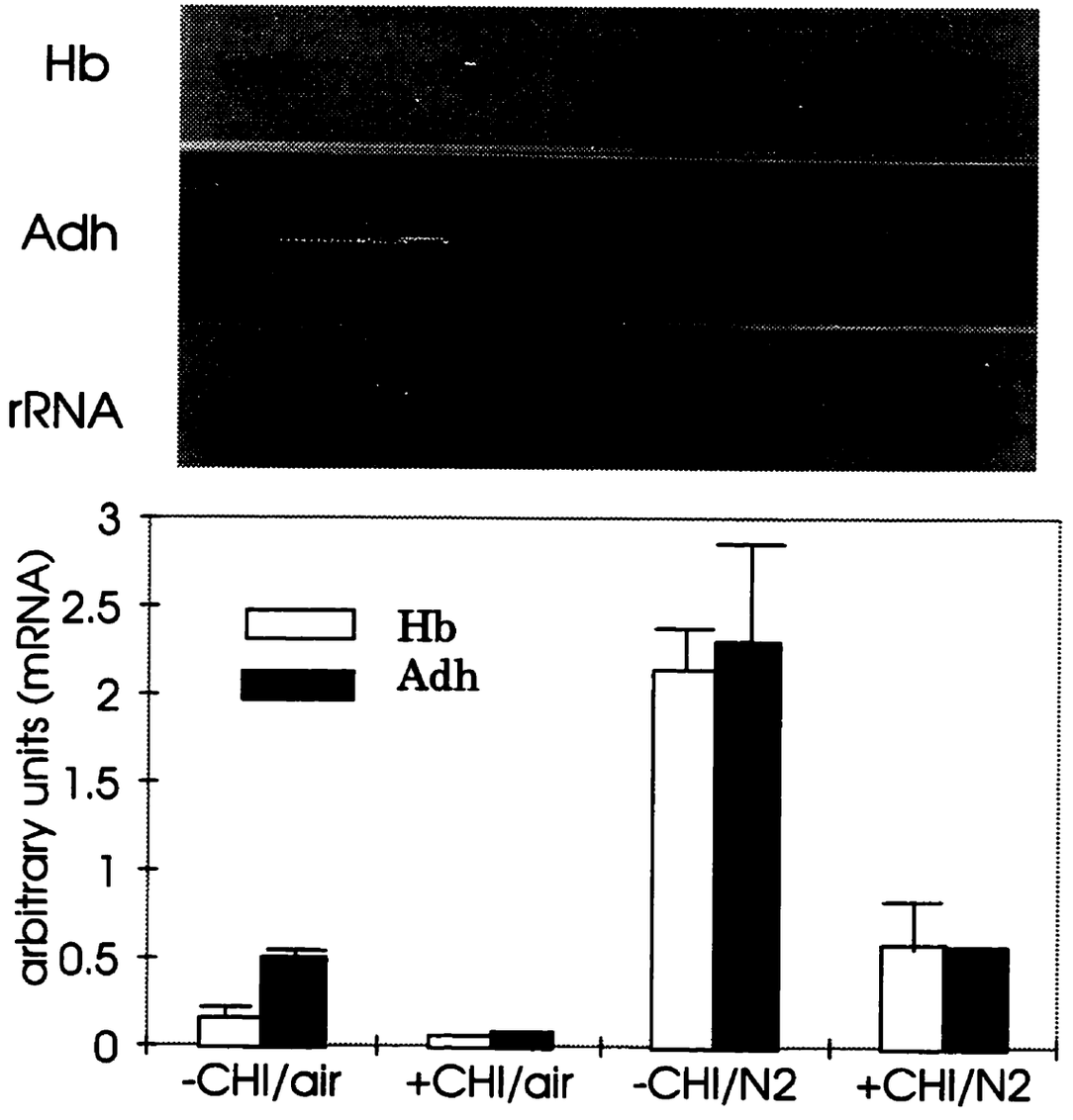
Cycloheximide (CHI) has been widely used to block protein synthesis in organisms. Twenty μg/mL of CHI has been reported to inhibit protein synthesis in barley aleurone layers by 95% (Liu & Hill, 1995). As demonstrated previously, Hb and *Adh* were induced by anoxia. However, this induction was greatly inhibited by CHI (Fig. 29). Anoxia treatment resulted in an approximately 13-fold increase in Hb gene expression and a 4-fold increase in *Adh* transcription over normoxia. However, in the presence of CHI, about 70% decrease in both *Adh* and Hb mRNA was noticed under anaerobic conditions, suggesting that *de novo* protein synthesis is required for Hb and *Adh* gene expression.

4.6. A role of Hb in barley aleurone layers and coleoptiles?

4.6.1. Effect of Hb-inhibitors on Hb gene expression

Some metal ions, such as Ni^{2+}, Co^{2+} and Mg^{2+}, have been demonstrated to be capable of replacing the iron atom of the heme moiety (Shibayama et al., 1986; Yonetani et al., 1974; Goldberg *et al.*, 1988). Thus, to investigate the impact of hemoglobin modification on Hb itself as well as *Adh* and *Ldh* gene expression, barley aleurone layers were incubated with Ni^{2+} and Co^{2+} separately followed with analysis of Hb, *Adh* and *Ldh* transcription in this tissue. Northern blot

Figure 29. Effects of cycloheximide on Hb and Adh gene expression. Barley aleurone layers were preincubated in water or cycloheximide (CHI) at a concentration of 20 µg/mL for 1 h. The layers were then set under N_2 or air for 6 h in the same solutions. Total RNA was isolated, and Northern blot analysis was carried out with a barley Hb probe, then a Adh probe and finally a rDNA probe as described in the "Materials and Methods". The signals from the blots were quantified and normalized as described in previous figures. The data shown below the blots are means $\pm$ SE values of four replicates.


Hb
Adh
rRNA
arbitrary units (mRNA)
3
2.5
2
1.5
1
0.5
0
Hb
Adh
-CHl/air
+CHl/air
-CHl/N2
+CHl/N2

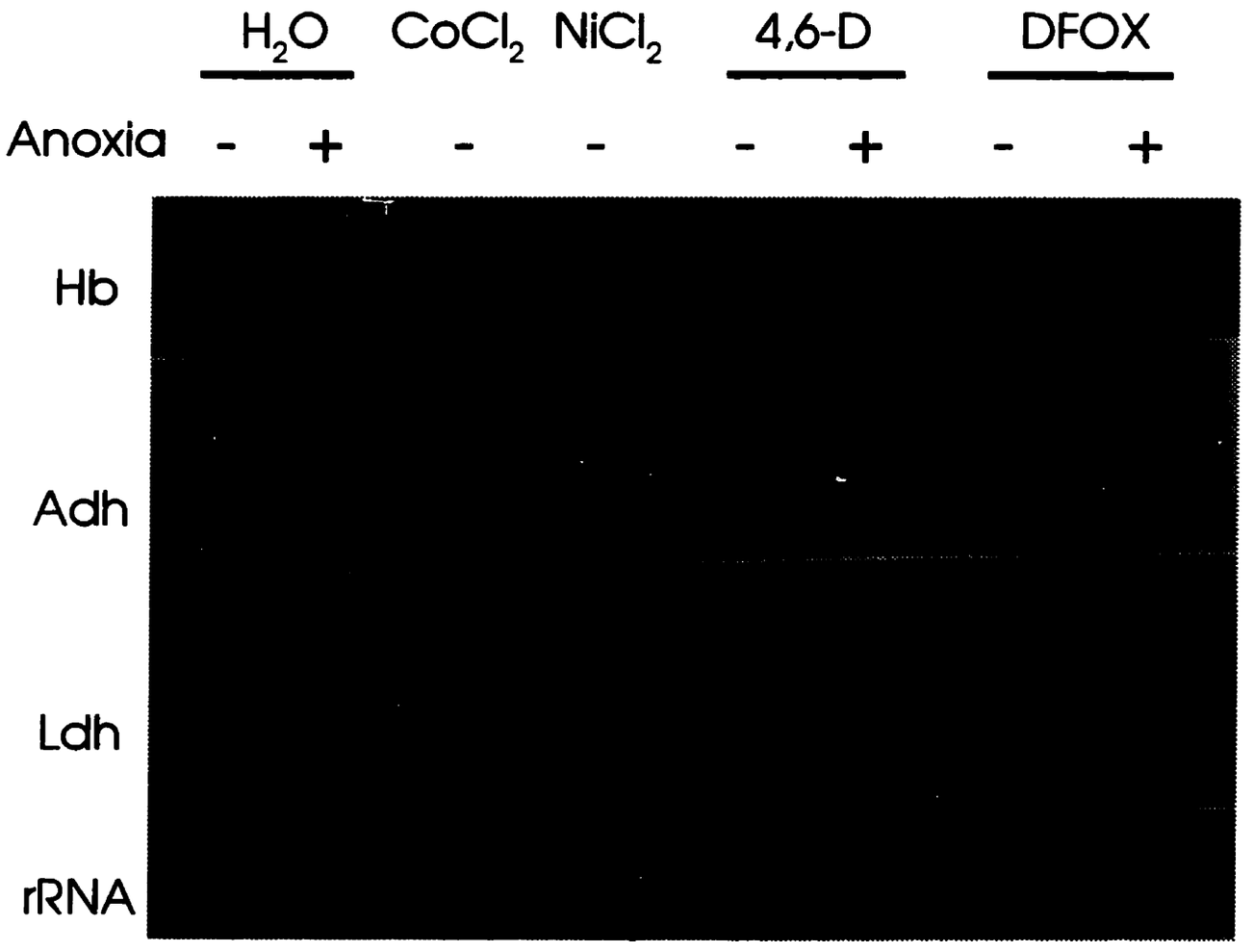
analysis (Fig. 30) indicated that nickel ions significantly increased Hb, *Adh* and *Ldh* transcription at levels comparable to anoxia, a typical condition under which Hb is greatly induced. Cobalt ion was much less effective on Hb, *Adh* and *Ldh* induction compared to Ni^{2+} and anoxia, although a slight stimulation of both Hb, *Adh* and *Ldh* was observable.

Heme synthesis from δ-aminolevulinate through the action of δ-aminolevulinic acid (ALA) dehydrogenase is inhibited by 4,6-dioxoheptanoic acid (succinylacetone, 4,6-D) (Tschudy *et al*, 1981), an ALA analog, and desferrioxamine (DFOX) (Shedlofsky *et al*, 1987), an iron chelator. These two chemicals were used to treat barley aleurone layers. From Fig. 30, it can be seen that both 4,6-D and DFOX stimulated Hb, *Adh* and *Ldh* gene expression even under aerobic conditions, although DFOX was much less effective than 4,6-D. These two compounds appeared to have no further stimulating or inhibitory effects on anoxia-induced gene expression of Hb and *Ldh*.

4.6.2. Effect of exogenous recombinant Hb on NADH oxidation in barley coleoptile mitochondria

To investigate whether Hb is involved in NADH oxidation in mitochondria either by accepting electron transmitted from NADH in the presence or absence of oxygen or by facilitating oxygen diffusion in the medium and thus accelerating NADH oxidation, recombinant barley hemoglobin was added to the mitochondrial medium together with exogenous NADH. No alteration in NADH oxidation rate was observed (data not shown).

Figure 30. Effects of Co^{2+}, Ni^{2+}, desferrioxamine and 4,6-dioxoheptanoic acid on Hb, Adh and Ldh gene expression in barley aleurone layers. Freshly prepared barley aleurone layers were preincubated with various additions for 1 h. The layers were then set under N_2 or air for another 6 h in the same solutions. Total RNA was isolated. Ten micrograms of RNA was electrophoresed in 1.25% denaturing agarose gel. Loading equivalence and RNA integrity were confirmed prior to the transfer as described in "Materials and Methods". RNA was blotted to Hybond-N^+ (Amersham, UK), and then probed with Hb, *Adh*1, *Ldh*1 and rDNA probes as described in Figure 16. Desferrioxamine (DFOX) was used at the concentration of 130 µM, 4,6-dioxoheptanoic acid (4,6-D) at 2 mM, $NiCl_2$ at 300 µM, $CoCl_2$ at 100 µM. '-' represents the layers that were incubated in air, while '+' represents the layers that were incubated in N_2.


H2O
CoCl2
NiCl2
4,6-D
DFOX
Anoxia - + - - - + - +
Hb
Adh
Ldh
rRNA

4.6.3. Effect of pre-treatment of barley seedlings under anoxia on respiration in coleoptiles

As shown in Fig. 9, Hb protein was enhanced by anoxia in barley coleoptiles. To investigate the possible impact of elevated level of Hb on oxygen consumption in this tissue, 2-d-old seedlings were pre-treated under anoxia for 24 h, when Hb protein levels were greatly enhanced, and oxygen uptake of coleoptiles was analysed after the seedlings were returned to air. Table V shows the results at various time points after the pre-treatment. Oxygen consumption in the anoxia-treated coleoptiles was lower than that in the un-stressed coleoptiles at the 0 time point. However, it increased to a level about 15 to 20% higher in the anoxia-treated than that in the untreated coleoptiles over the remaining incubation period. These results suggest that anaerobic stress elevates respiration in the form of oxygen consumption. However, this does not necessarily mean that the stimulated oxygen uptake is due to elevated hemoglobin content in the cells.

Table V. ***The effects of anoxia on oxygen consumption in barley coleoptiles***

Two-d-old barley seedlings (the coleoptiles were approximately 2 cm in length) were set either under N_2 or under air in darkness at room temperature for 24 h. After the treatment, the seedlings were placed under normal air atmosphere. Oxygen consumption of coleoptiles was measured subsequently. The data are means of two replicates.

Time point in air (min) after Pre-treatment	Primary treatment (24h)	O_2 uptake	
		(nmole/g.f wt/min.)	% the control (air)
0	Air	129.5	100
	N_2	80.4	62.1
20	Air	129.9	100
	N_2	153.7	118.3
40	Air	\	\
	N_2	150.1	\
60	Air	126.4	100
	N_2	154.7	122.0
120	Air	145.6	100
	N_2	168.5	115.8
240	Air	157.1	100
	N_2	188.3	119.8

5.Discussion

5.1. Hb occurrence in the plant kingdom

The discovery of hemoglobin in cereal plants is consistent with the hypothesis proposed by Appleby et al. (1988) that hemoglobin exists in all plant species. It is now generally accepted that the known animal and plant Hbs evolved from a common ancestor about 1.4 billion years ago (Hardison, 1996). Although leghemoglobins share a high homology with non-leguminous hemoglobins, the nucleic acid cross hybridization was very poor, leading to the early assumption that hemoglobin exclusively exists in leguminous plants (For reference, see Appleby, 1984). Soybean leghemoglobin nucleic acid sequences were reported to have successfully hybridized with several actinorhizal plants (Roberts et al., 1985), but, such reports have been questioned by other researchers (Appleby et al., 1988). Indeed, in many cases, leghemoglobin DNA sequences failed to cross-hybridize with their counterparts in non-legumes, such as cereal plants (Taylor et al., unpublished data). Thus, the accidental discovery of hemoglobin in barley and other cereal plants (Taylor et al., 1994) was a remarkable event in hemoglobin research, which strengthened the earlier hypothesis that hemoglobin may be widely distributed in the plant kingdom (Appleby et al., 1988). The recent discovery of a non-symbiotic hemoglobin in soybean (Andersson et al., 1996), a typical leguminous plant species, further supports this hypothesis. It is possible that leghemoglobin evolved to a nodule-specific macromolecule during the emerging period of legumes. Indeed, to date, all identified plant hemoglobins, regardless of their symbiotic or non-symbiotic nature, share the same gene structures, i.e., three introns located at identical sites of the gene (Anderson et al., 1996; Guy et al., 1997). Non-symbiotic hemoglobin may therefore be more ancient than symbiotic hemoglobin.

Southern blot analysis (Fig. 3) clearly showed that the barley hemoglobin gene can cross-hybridize with genomic DNA from different monocotyledonous species including rye, wheat, maize, oat and rice. It was suggested that low number of Hb gene copies may exist in barley, rye and maize because a single hybridization band observed in each restricted DNA lane (Taylor et al., 1994). The results shown in Fig. 3 supports the conclusion that low gene copy numbers are present in cereal species (Taylor et al., 1994), except rice, in which three to four hybridization bands were observed. Indeed, two closely-related but distinct hemoglobins have been reported in rice (For references, see Andersson et al., 1996). The multiple bands may thus indicate the presence of a gene family or multiple gene copies in rice.

According to Andersson *et al.* (1996), barley hemoglobin and soybean non-symbiotic hemoglobin are much closer to each other than to legume symbiotic hemoglobins on a protein sequence basis. Failure of cross-hybridization using leghemoglobin DNA probes in non-legume-sourced DNA does not rule out the possibility that barley Hb DNA probe may be able to hybridize with DNA from dicots, including legumes. An attempt to demonstrate this is shown in Fig. 3b. The results indicate that barley Hb DNA sequences can hybridize to one band in the genomic DNA isolated from bean, a legume; one faint band is also present in the DNA isolated from *Brassica*, a non-leguminous dicot. The results suggest that barley hemoglobin gene-like sequences, maybe the non-symbiotic hemoglobin, exist in dicots including legume species. These observations are consistent with the findings that non-symbiotic soybean hemoglobin shares high similarities with barley hemoglobin (Andersson et al., 1996). However, in another separate experiment, one blurred hybridization band was present in pea, and no apparent bands were observed in white clover and alfalfa (Fig. 3b). This failure may be due to the high-stringency

washing after the hybridization. Obviously, more carefully controlled hybridization is necessary in order to clarify the findings.

5.2. Hb gene expression and ATP availability

Previous studies have indicated that non-symbiotic Hb in plants is tissue specific (Appleby et al., 1988). *Parasponia* Hb promoter directed gene expression in transformed tobacco is mainly located at root tips and within the stele of the root (Boguz et al., 1990), where relatively higher metabolism occurs. In barley, Hb transcript is found in roots, coleoptiles and aleurone layers (Taylor et al., 1994; Fig. 4). Since all the tissues mentioned above may suffer oxygen deprivation to a certain extent during plant growth and development, one would expect that Hb gene expression might be oxygen related. Indeed, Hb gene expression is up-regulated by low oxygen stress in bacteria (Wakabayashi et al., 1986) as well as in maize roots, barley roots and aleurone layers (Taylor et al., 1994; Fig. 5 to Fig. 9). When oxygen concentration was decreased to 5 -10%, Hb transcript levels were enhanced. A dramatic increase was observed as oxygen concentration was decreased further to 0%. Like some identified anaerobic-response-polypeptides (ANPs), Hb transcripts started to increase within a very short period of time under anoxia in barley aleurone layers (Fig. 6a). Moreover, Hb protein level was also increased under anaerobic conditions (Fig. 7). Thus, Hb in addition to ADH and LDH should be considered as an anaerobic response protein.

Regulation of ANPs differs from gene to gene. Typically, both transcripts and polypeptides, such as those of alcohol dehydrogenase and lactate dehydrogenase, are up-regulated by oxygen deprivation (Hanson & Jacobson, 1984). However, both transcriptional and

translational regulation occur in several anaerobic responsive genes (Ricard et al., 1996). For instance, sucrose synthase mRNA levels are dramatically enhanced by oxygen deficit whereas no significant increase in the protein is observed (McElfresh & Chourey, 1988). Hb messenger level was enhanced by low-oxygen stress as much as 50 fold (Fig. 5 to Fig. 30), however, only 2- to 3-fold increase was observed at the protein level (Fig. 7, Fig. 8), indicating that translational regulation of Hb gene expression may exist.

Several anaerobic-response genes show a transient induction when the plant tissues are subjected to low oxygen stress (Sachs et al., 1980). In maize roots suffering from waterlogging, the induction of Hb appeared to be less pronounced than in barley roots and aleurone layers (Silva-Cārdenas, 1997; Fig. 11 and Fig. 12). This suggests that different mechanisms may exist to direct the Hb gene expression in barley and maize roots subjected to low oxygen tension. Interestingly, both Hb transcript and protein levels decreased once oxygen deficit was removed (Fig. 8), suggesting that Hb gene expression in barley aleurone layers is low-oxygen dependent and closely regulated in tissues. Moreover, anoxia treatment of leaves, the tissues where no Hb transcripts were observed under normal growth conditions (Taylor et al., 1994; Fig. 4), appeared to induce a Hb-size band in the RNA hybridization blot (Fig. 10). This observation, however, has to be treated cautiously since a large amount of RNA was loaded in the RNA gel and also apparent degradation of RNA occurred due to the oxygen deprivation injury in leaves during the treatment.

To investigate why Hb is induced under oxygen limited conditions, especially with energy metabolism, a series of experiments related to mitochondrial respiration were conducted (Fig. 16 to Fig. 21).

CO binds tightly to plant hemoglobins (Gibson et al., 1989), but less so to cytochromes (for reference, see Voet & Voet, 1990). In the initial experiments, we designed treatments in which we tried to minimize CO effects on cytochromes by using relatively low concentrations of CO. Estimates in leaf tissue suggest that cytochrome *c* oxidase may be insensitive to CO at ratios of $CO:O_2$ below 40:1 (Naik et al., 1992). Assuming this is so in barley aleurone layers, the observed effects of CO on Hb gene expression would likely to be attributed to the binding of CO to hemoglobin. The results shown in Fig. 16 indicate an increase in hemoglobin gene expression in the presence of CO. Since there are no direct data regarding the CO effects on cytochromes and Hb in barley, it is still possible that this induction is due to the effects of CO on mitochondrial respiration. If this is the case, interfering with mitochondrial respiration would induce Hb gene expression as well. Indeed, the results with other respiratory inhibitors do indicate that interference with mitochondrial respiration and ATP synthesis strongly induces hemoglobin gene expression.

Cyanide, an inhibitor of complex IV in the mitochondrial electron transport chain, and also a ligand of hemoglobin, increased the level of hemoglobin transcripts in barley aleurone layers (Fig. 17a). Interestingly, in mammalian systems, cyanide has neither inhibitory nor inductive effects on known anaerobic responsive genes, such as erythropoietin (*Epo*) and *Ldh* (Firth et al. 1994; Ebert et al., 1996). It appears that the responses to cyanide in barley aleurone layers are different from those in mammalian systems. Antimycin A, an inhibitor of complex III in the electron transport chain, enhanced the level of hemoglobin mRNA levels in barley aleurone layers (Fig. 17b), too. Thus, the results with antimycin A indicate that the effect is not due to interference with oxygen binding to a heme structure. Very similar results are reported in

Saccharomyces cerevisiae (Zhao et al., 1996). 2,4-Dinitrophenol, which stimulates electron transport and thus increases oxygen consumption (Douce, 1985; Table II) in the absence of ATP synthesis, also increased hemoglobin expression (Fig. 18). Oligomycin, which specifically inhibits oxidative phosphorylation in mitochondria by inhibiting ATP synthase through binding to the F_o subunit and interfering with H^+ transport through F_o (Voet and Voet, 1990), also increased Hb expression (Fig. 19) without significant effects on electron transport and oxygen consumption (Table III). The induced hemoglobin expression, therefore, was not the result of inhibited electron transport or the accumulation of reducing equivalents in the tissue. Conditions under which there was a 37% reduction (antimycin A and cyanide, respectively), an 86% stimulation (2,4-dinitrophenol) or no change (oligomycin) in oxygen uptake all permitted induction of Hb mRNA, making it unlikely that mitochondrial O_2 availability is directly involved in the induction process. Taken together, the results suggest that ATP is a critical component in hemoglobin induction.

As demonstrated elsewhere (Siedow & Umbach, 1995), the alternative pathway functions once the cytochrome pathway is saturated or blocked. However, this pathway is much less efficient in ATP production than is the cytochrome pathway. Thus, blocking the alternative pathway alone should have little impact on Hb gene expression if ATP is a major regulatory factor in Hb induction as discussed above. BHAM, an inhibitor of alternative oxidase, slightly increased Hb transcripts (Fig. 20). However, the effect was not as strong as with KCN alone or KCN plus BHAM. Like desferrioxamine (DFOX), which appeared to induce Hb slightly (Fig. 30), BHAM is an iron chelator, and may, therefore, act by interference with iron-requiring processes rather than on electron transport or ATP production. It may be worthwhile to mention that the alternative pathway was working along with the cytochrome pathway in barley aleurone layers even under

normal incubation conditions, because BHAM alone reduced O_2 uptake by 17% (Table IV).

It has been shown that a close relationship exists between energy charge and the rate of ethanol plus lactate production in plants (Saglio et al., 1980). Seeds storing lipids as the main carbon reserve have been observed to have 20 to 50 times the rate of ethanol production during anaerobiosis compared to those containing starch (Al-Ani et al., 1982). The energy charge of the starchy seeds after several hours of anoxia was greater than 0.6 while the lipid-containing seeds maintained energy charges of less than 0.35. Johnson et al. (1989) have shown that maize roots exposed to a period of hypoxia, prior to experiencing anoxic conditions, remain viable for longer periods of time. They attribute this to the induction of *Adh*, providing ATP synthesis through anaerobic fermentation. Our results would suggest that the induction of hemoglobin synthesis, triggered by a transient decline in energy charge, may allow a period of oxidative metabolism during hypoxia to generate ATP for the synthesis of enzymes for fermentative metabolism.

If this assumption were correct, one would expect that Hb transcription might be stimulated by nitrate. An appropriate concentration (around 10 mM) of nitrate slightly enhanced Hb transcription under aerobic conditions, and increased the transcription by 40% under anaerobiosis (Fig. 21). Nitrate reduction is an energy consuming process in which 8 electrons are required to reduce one NO_3^- molecule to one NH_4^+ molecule (Salisbury and Ross, 1985). The first step for the reaction is from nitrate to nitrite, which is catalyzed by nitrate reductase, a substrate inducible enzyme (Ferrari & Varner, 1970). Although nitrate reduction is indirectly linked with energy metabolism, the demand for NADH in the nitrate reduction process may result in less ATP production in the tissue. However, nitrate reductase is a highly regulated enzyme, and the activity in root tissue increases rapidly upon depletion of oxygen, where ATP levels are low

and AMP levels are high (Glaab & Kaiser, 1993). Inhibition of mitochondrial respiration with antimycin A under aerobic conditions results in increased nitrate reductase activity, which is comparable to that under anaerobiosis in barley aleurone layers (Ferrari & Varner, 1970). The relatively small effect of nitrate on hemoglobin gene expression in air and a slight increase under anaerobic conditions (Fig. 21) are likely due to a combination of increased nitrate reductase synthesis and regulation of nitrate reductase activity. Under aerobic conditions, nitrate reductase would be synthesized due to the presence of nitrate, but its activity would be low as a result of higher ATP levels. With anaerobiosis, nitrate reductase activity would be stimulated, resulting in competition for NADH with ADH and LDH. With time, ATP levels may decline further due to a lack of fermentative ATP synthesis. The decline of hemoglobin gene expression at high nitrate concentrations (Fig. 21) is likely a consequence of accumulation of cytotoxic accumulation of nitrite (Kaiser & Huber, 1994).

5.3. Hb gene expression and signal transduction

It has been shown that cytosolic calcium is involved in many physiological processes and gene regulation ranging from environmental stimuli to developmental cues. Anoxia-induced cytosolic Ca^{2+} fluxes and their impacts on cellular injury have been well studied in animal systems (Gasbarrinal et al., 1992a & b). Several reports regarding similar events in plant systems have emerged in recent years (Subbaiah et al., 1994a & b; Kuo et al., 1996). Maize seedlings treated with ruthenium red, an organella Ca^{2+} channel blocker, show a poor recovery after a brief anoxia treatment (Subbaiah et al., 1994a). This has been attributed to the abolition of an anoxia-induced Ca^{2+} influx ($[Ca^{2+}]i$) in the presence of ruthenium red. Ca^{2+} influx, thus, is considered as an early

signal transducer of oxygen depletion in the environment (Subbaish et al., 1994a & b). Indeed, maize cells have been shown to respond to anoxia by elevating $[Ca^{2+}]_i$ within a minute or two (Subbaiah et al. 1994b). The observation of ruthenium red (RR) decreased anoxia-induced Hb and *Adh* transcripts in barley aleurone layers (Fig. 22) is consistent with the results reported in maize seedlings, suggesting that a similar mechanism exists in both tissues in response to oxygen deprivation.

Further evidence of Ca^{2+} involvement in anaerobiosis of barley aleurone cells comes from experiments involving the divalent ionophore A23187 and the calcium chelator EGTA. As shown in Fig. 23 and Fig. 24, A23187 combined with EGTA abolished anoxia-induced Hb and *Adh* transcription. However, this inhibitory effect was removed by exogenous Ca^{2+}, but not Mg^{2+} (Fig. 24). Since A23187 perturbs ion-channel-controlled divalent cation movement in cells, cellular Ca^{2+} could enter the cell through the plasma membrane, even though Ca^{2+} influx is believed to arise from internal stores in maize cells facing anoxia (Subbaiah et al., 1994b). Interestingly, although Ca-dependent calmodulin (CaM) acts along with $[Ca^{2+}]_i$ in many processes such as mechanical stimuli and red/far-red responses in plants (Bowler & Chua, 1994), CaM appears not to participate in the response of Hb and *Adh* to anoxia in barley aleurone tissue. The CaM antagonist, W-7, failed to show inhibitory effect on anoxia-induced gene transcription except at a concentration of 1000 μM (Fig. 25), the highest concentration tested in this investigation.

Signal transduction is a very complicated cascade with the involvement of numerous enzymatic reactions (Bowler & Chua, 1994). Ca^{2+} influx in the cytoplasm is an early event in many signal transmission processes, including anoxia signal transduction (Subbaiah et al., 1994b;

Bush, 1995). It is logical to assume that there is a chain of reactions in which reversible protein phosphorylation(s) catalysed by specific protein kinase(s) and protein phosphatase(s) may subsequently occur. Wheat aleurone layers treated with okadaic acid, a protein phosphatase inhibitor, fail to respond to GA in many aspects, including rapid changes in cytosolic Ca^{2+} (Kuo et al., 1996), indicating that a protein phosphatase plays a role in GA signal transduction. Interestingly, this inhibitor has been demonstrated to be unable to interfere with anaerobic responses in the form of *Adh* induction and cytosolic Ca^{2+} changes. An OA-sensitive protein phosphatase, therefore, has been suggested to be a GA specific response (Kuo et al., 1996). The results shown in Fig. 28, however, disagree with the above suggestion. Anoxia-induced Hb and *Adh* transcription was inhibited by OA in a dose-dependent manner. There was an approximately 30%, 80% and 93% reduction in Hb mRNA by 10, 100 and 200 nM of OA, respectively, and an approximately 63%, 90% and 99.9% reduction in *Adh* transcripts by 10, 100 and 200 nM of OA, respectively, also.

Although similar patterns were observed in *Adh* and Hb gene expression under various conditions, different responses were observed when barley aleurone layers were subjected to A3 treatment under anoxia. The fact that A3 decreased anoxia-induced *Adh* transcription in a dose-dependent manner (Fig. 27) suggests that an A3-sensitive protein kinase(s) is also involved in anoxia signal transmission leading to *Adh*1 expression under oxygen deficit conditions. Thus, the cascade leading to *Adh* gene activation under anaerobiosis is composed of a series of reactions in which at least one OA-sensitive protein phosphatase and one A3-sensitive protein kinase are involved. Interestingly, the signal leading to Hb gene expression under anoxia appears to be independent of A3-sensitive protein kinase since no significant effects were observed in various

A3 treatments (Fig. 27). Multiple anoxia signal transduction pathways are likely to exist in the barley aleurone system. It is possible that the same signal detection and transmission mechanism occurs at an early stage of anoxia, and is followed by several signal by-passes which lead to more specific responses including activation of a specific individual gene like Hb or *Adh1*.

Inhibition of protein synthesis has been shown to block the expression of certain genes responding to a stimulus (Liu & Hill, 1995; Fig. 29). For instance, barley α-amylase/subtilin inhibitor (BASI) induced by ABA in barley aleurone layers is inhibited by the presence of cycloheximide (CHI), a protein synthesis inhibitor, whereas *Em* is not affected by CHI (Liu & Hill, 1995). In our experiments, CHI significantly decreased anoxia-induced Hb and *Adh* mRNA levels. However, it remains unknown whether this effect is due to decreased transcription rates or increased transcript turnover. It is possible that a newly synthesized protein is involved in the signal transduction leading to *Hb* and *Adh* gene expression. Inhibiting the synthesis of this protein will, therefore, block the signal transmission; consequently, Hb and *Adh* expression is blocked.

Although Ca^{2+} is apparently involved in Hb and *Adh* activation without the participation of CaM under anaerobiosis, it remains unexplained why RR appears to induce *Adh*, *Ldh* and even Hb to a certain extent under aerobic conditions (Fig. 22). W-7, A3 and OA apparently increased Hb and *Adh* gene expression under normoxia (Fig. 25, Fig. 27 & Fig. 28), too. It is possible that disturbance of calcium ions or CaM under normal aerated conditions may subsequently trigger a series of responses including gene activation. If this statement is correct, it would also fit the observation that A3 and OA stimulated Hb and *Adh* transcription under aerobic conditions (Fig. 27 & Fig. 28). It has been established that *Adh* responds to multiple stimuli, such as dehydration, cold shock (Dolferus et al., 1994) and the phytohormone ABA (de Bruxelles et al., 1996) in

various plant species. It is possible that Hb might also be induced by multiple stimuli. An alternative signal detection and transduction pathway, which differs from the anoxia signal transmission pathway, initiated by interfering with calcium balance between cytoplasm and Ca^{2+} pool, may exist in barley aleurone cells. In this pathway, CaM may be a negative factor to suppress the activity of the signal cascades that leads to the activation of anaerobic responsive genes including Hb and *Adh*. An A3-sensitive protein kinase and an OA-sensitive protein phosphatase might be involved in this pathway as well.

Although G-proteins have been suggested to play a role in elicitor induced rapid oxidative burst in soybean cells (Legendre et al., 1992) and phytochrome-mediated *cab* gene activation (Romero & Lam, 1993), cholera toxin, a G-protein activator, failed to mimic anaerobic stress in barley aleurone layers and protoplasts (Fig. 26). However, this does not rule out the possibility that G-proteins may play a role in the signal transduction process as mentioned in the "results" section. Caution should be used in this kind of experiments since cholera toxin is a two-subunit-protein with a molecular weight around 35 kD, and may have difficulty passing cell walls, especially intact tissue like the aleurone layer.

5.4. Function of hemoglobin

It has been proposed that Hb may function as an oxygen sensor to monitor oxygen level in the tissues and, when necessary, to switch the metabolism from an aerobic to an anaerobic pathway in plant roots (Appleby et al., 1988). In *E. coli*, the flavohemoglobin (Hmp) has also been suggested to act as an oxygen sensor (Poole et al., 1994). Some evidence suggests that at least some oxygen sensors are hemeproteins in rhizobial (Gilles-Gonzalez et al, 1991; Gilles-

Gonzalez & Gonzalez, 1993) and mammalian systems (Goldberg et al., 1988). The structural conformation of these hemeproteins appears to be critical for them to work as oxygen signal molecules. There are two conformations, oxy and deoxy forms, existing in hemoglobin in living tissues (for reference, see Wittenberg & Wittenberg, 1990). The deoxy form, the conformation of the molecule when it is deprived of O_2, has been proposed to serve as a signal transducer and trigger anaerobic responses (Goldberg et al., 1988). The binding of Hb to CO maintains the Hb molecule in an oxy conformation even in the presence of hypoxia, thus preventing the induction of anaerobic responsive genes (e.g., *Epo*) under hypoxia in mammalian systems (Goldberg et al., 1988). However, the results in Fig.16 showed that carbon monoxide appears not to prevent *Ldh*, *Adh* and Hb transcription under hypoxia. Moreover, the compound itself induced all the tested anaerobic genes to a certain degree under normoxia.

The induction of the hemoglobin gene under low O_2 conditions shares some similarities with that observed for alcohol dehydrogenase and lactate dehydrogenase which are involved in anaerobic metabolism (Fig. 5 and Fig. 6; Taylor et al., 1994). The *Hb* mRNA induction parallels rather than anticipates the expression of the *Adh* and *Ldh* transcripts. This would not be expected if the only function of the protein were as an oxygen sensor triggering the anaerobic response and thus the increased expression of these two genes.

Interference with heme biosynthesis or heme function by chelating or replacing the iron atom, a functional part of the heme, has been shown to block hypoxia-induced expression of erythropoietin, a hormone that is responsible for red cell and hemoglobin production in humans (Goldberg et al., 1988). It would be reasonable to expect that anoxia-induced transcription of *Adh* and *Ldh* is inhibited by blocking heme biosynthesis or heme function, if hemoglobin acts as an

oxygen sensor in barley aleurone cells. However, neither the heme biosynthesis inhibitor (4,6-dioxoheptanoic acid) nor the iron chelator (desferrioxamine) appeared to affect anoxia-induced gene expression of *Adh*, *Ldh* and Hb under anoxia (Fig. 30). Moreover, 4,6-D and DFOX stimulated their expression even under aerobic conditions. It is unlikely that Ni^{2+} increased Hb, *Adh* and *Ldh* gene expression (Fig. 30) by locking hemoglobin in its deoxy form and consequently initiating anaerobic responsive genes even though both Ni^{2+} and Co^{2+} have been suggested to be capable of replacing the iron atom in the middle of heme moiety (Ikeda-Saito et al., 1981; Goldberg et al., 1988). Taken together, the evidence suggests that hemoglobin in barley aleurone layers is unlikely to function as an oxygen sensor to detect and trigger anaerobic metabolism when the tissue is subjected to low oxygen stress.

Expression of *Vitreoscilla* hemoglobin in *Saccharomyces cerevisiae* shows that the microbial molecule is linked to respiration and electron transport (Chen et al., 1994). Studies on bacteria indicate that hemoglobin biosynthesis increases dramatically when oxygen availability is low (LaCelle et al., 1996). So, it has been suggested that *Vitreoscilla* hemoglobin functions as an oxygen storage-trap to supply oxygen to the terminal oxidase under hypoxic conditions (Wakabayashi et al., 1986; Dikshit et al., 1989). Recombinant *E.coli* cells containing *vgb* (VHb gene) grew faster and to greater cell densities than cells containing a comparable plasmid, and the VHb increased the rate of oxygen use when dissolved oxygen concentration was less than 5% of atmospheric (Kallio et al., 1994). It is possible that the plant Hb in barley aleurone tissue works as an oxygen storage-molecule to improve oxygen supply when the oxygen concentration falls below a critical level. Energy status is involved in the process. When the production of ATP slows down, the cell will start synthesizing more Hb to trap more oxygen and thus to improve oxygen

availability for mitochondrial respiration. Increased amounts of hemoglobin, then, probably could elevate ATP production efficiency by providing more oxygen as suggested in *E.coli* containing VHb (Kallio, et al., 1994). If this assumption is correct, elevation of Hb protein in barley tissue such as coleoptiles would thus be expected to increase O_2 consumption. Indeed, in a preliminary experiment, when young seedlings were treated under anoxia and returned to normal air atmosphere, increased O_2 uptake was observed (Table V). However, this observation could not be used simply as evidence to support the hypothesis since cellular injuries may also cause oxygen uptake enhancement in the tissue (for reference, see Salisbury & Ross, 1985). Nevertheless, the oxygen storage or facilitator theory about barley Hb is challenged by the observations of O_2 association and dissociation rates of recombinant barley hemoglobin (Duff et al., 1997). This hemoglobin has an unusual high O_2 association rate (9.5×10^{-6} $M^{-1}.S^{-1}$) with a very low O_2 dissociation rate (0.0272 s^{-1}). It would, thus, be difficult for the molecule to facilitate the diffusion of O_2 to the mitochondria, where O_2 is apparently needed and consumed. A complex of hemoglobin with another protein or co-factors may differ significantly from a hemoglobin alone in many ways including O_2 binding-releasing characteristics. Indeed, it appears that such a protein, which is capable of association with barley hemoglobin, exists in barley seed extracts (Duff, personal communication). However, any speculation should be treated cautiously before the protein is isolated and characterized.

Vitreoscilla hemoglobin has been proposed to act as a terminal oxidase (Dikshit et al., 1992). Indeed, some hemoglobins including *Vitreoscilla* Hb and *E.coli* Hb possess flavin domains, which apparently have some redox characteristics. *Vitreoscilla* Hb has also been found to support the aerobic growth of *E.coli* lacking terminal oxidase (Dikshit et al., 1992). If barley hemoglobin

functions as a terminal oxidase or at least as a redox protein, it would be reasonable to expect that at least part of the protein might be located in the mitochondria. Studies on *Saccharomyces cerevisiae* transformed with *Vitreoscilla* Hb have indicated that approximately 40% of the Hb is located in mitochondria (Chen et al, 1992), and the rest is cytoplasmic. However, it was apparent that the Hb in barley was mainly found in the cytoplasm rather than in mitochondria in anoxia-induced coleoptiles (Fig. 15). Thus, the protein is unlikely to be a part of the mitochondrial electron transport chain. Again, it would be hard to explore whether this protein is a terminal oxidase or a redox protein without any knowledge of its possible association with other proteins or co-factors, since such an association may significantly alter the properties of Hb.

6. Summary

This study shows that barley Hb-like DNA sequences exist in different monocots and dicots, indicating that the Hb gene is widely distributed in the plant kingdom. Northern analysis shows that Hb gene expression is tissue specific under normal cultivating conditions. Hb mRNA is induced when O_2 tension decreases to 5-10%. Both Hb messenger and protein are induced by oxygen deficit within a short period of anaerobiosis. Interfering with mitochondrial respiration or ATP production results in Hb induction, suggesting that Hb gene expression is likely to be regulated by ATP level or energy charge. Hb gene expression is inhibited in the presence of a calcium chelator EGTA and a divalent ionophore A23187. However, this inhibition is removed if exogenous Ca^{2+} is added subsequently, suggesting that calcium is involved in Hb gene regulation. Okadaic acid, a protein phosphatase inhibitor, blocks anoxia-induced Hb transcription, and A3, a protein kinase inhibitor, fails to do so. These results may suggest that an OA-sensitive protein phosphatase is involved in Hb gene regulation and signal transduction process. The physiological functions of hemoglobin in barley remain unclear.

7. References

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Appendix I. Major Solutions

TE (pH 8.0)

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

SSC (20 x)

3 M NaCl

0.3 M Na_3 citrate

Denhardt's solution (100x)

2% (w/v) Ficoll

2% (w/v) PVP (Polyvinylpyrrolidone)

2% BSA (bovine serum albumin)

TBS (Tris-buffered saline, pH 7.4)

0.8% (w/v) NaCl

0.02% (w/v) KCl

0.3% (w/v) Tris

0.0015% (w/v) phenol red

TTBS (pH 7.4)

TBS

0.05% Tween 20

CTAB buffer

2% CTAB (cetyltrimethylammonium bromide, w/v)

100 mM Tris (pH 8.0)

20 mM EDTA (pH 8.0)

1.4 M NaCl

1% PVP (polyvinylpyrrolidone)

TAE buffer

0.04 M Tris-Acetate

0.001M EDTA

10 x RNA gel-running buffer

0.2 M MOPS (3-(N-morpholino)propanesufonic acid, pH 7.0)

80 mM Na-Acetate

5 mM EDTA (pH 8.0)